DEVELOPMENT AND CHARACTERIZATION OF AN IN-VITRO TISSUE CULTURE MODEL FOR EQUINE LAMINITIS

A Thesis

by

MARGARETE DAISY JOHNSON

Submitted to the Office of Graduate and Professional Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

MASTER OF SCIENCE

Chair of Committee,	Bhanu Chowdhary
Co-Chair of Committee,	Jane Welsh
Committee Members,	Jan Janecka
	Stephen Smith
Head of Department,	Evelyn Tiffany-Castiglioni

August 2014

Major Subject: Biomedical Sciences

ABSTRACT

Equine laminitis, a disease affecting the laminar tissue in the hoof, is a common and debilitating disease in horses with a significant impact on the equine industry. Currently nearly all laminitis studies are conducted in live horses, a process that is both expensive and limited in biological replicates. Thus the development of an in vitro model for the disease is an important step in advancing laminitis research. Recent evidence suggests that apolipoprotein A-IV (apoA-IV) may be involved in the chronic form of the disease but little is known about this protein in the horse, and its effects on the laminar tissue are unknown. The primary goal of this project was to produce a model for inducing inflammation in slices of laminar tissue in culture. We tested two inflammatory agents: interleukin 6 (IL-6) and lipopolysaccharide (LPS) and measured their effect on the expression of inflammatory cytokines and seven laminitis-associated genes found to be differentially expressed in horses with induced laminitis. The second goal of the project was to test the effects of apoA-IV on laminar tissue inflammation in our model in the presence and absence of the two inflammatory agents, and to further characterize the protein in horses by determining its sequence and expression pattern in this animal.

The laminar tissue remained alive and contamination-free over the course of the experiment, showing the viability of our culture. IL-6 did not induce changes in gene expression consistent with those found in horses with laminitis. However, the addition of LPS led to changes in cytokine expression mimicking those seen in horses with induced laminitis and increased two of the seven laminitis-associated genes. The addition of apoA-IV had no effect on laminar tissue inflammation by itself or in the presence of IL-6 or LPS. We

found the highest expression of APOA4 in the liver followed by the small intestine, a pattern unique in its high hepatic contribution. A better understanding of how apoA-IV is produced and functions in horses may shed light on its role in laminitis. In the future our tissue culture model could be used in testing agents suspected of causing laminar tissue inflammation and eventually in the development and testing of potential treatments for laminitis.

DEDICATION

This thesis is dedicated to my parents, Susan and Lee, for all their help and support over the course of my master's work.

ACKNOWLEDGEMENTS

I would like to express many thanks to my committee chairs, Dr. Welsh and Dr. Chowdhary, and to my committee members Dr. Janecka and Dr. Smith for all of their advice and support throughout my thesis project. I am particularly thankful for all of the help with data analysis, writing, and administrative tasks from Dr. Welsh, who performed much of this work before becoming a co-chair of my committee. I would also like to thank my labmates for all of their support during my time here. I am especially grateful for all of the input and support provided by Dr. Steelman from the initial design phase through the final writing process of this project. She has truly been my mentor during my master's project. I would also like to thank Dr. Dangott for his enormous help with my protein work, much of which could not have been completed without his help and advice. Finally, I would like to thank my parents for all of their support and patience during the undertaking of my master's degree.

NOMENCLATURE

ACTG	Gamma actin
ADAMTS1	A disintegrin and metalloproteinase with thrombospondin motifs 1
ApoA-IV	Apolipoprotein A-IV (protein)
APOA4	Apolipoprotein A-IV (gene)
BSA	Bovine serum albumin
CCL2	Chemokine (C-C motif) ligand 2
CD14	Cluster of differentiation 14
CXCL14	Chemokine (C-X-C motif) ligand 14
DEFB4	Beta-defensin 2
IFN-γ	Interferon gamma
IL-1β	Interleukin-1β
IL-6	Interleukin-6
IL-8	Interleukin-8
LBP	Lipopolysaccharide binding protein
LPS	Lipopolysaccharide
MMP	matrix metalloproteinase
SOD2	Superoxide dismutase 2, mitochondrial
S100A8	S100 calcium binding protein 8
TLR4	Toll-like receptor 4
TNF-α	Tumor necrosis factor alpha

TABLE OF CONTENTS

Page

LIST OF FIGURES

Page

LIST OF TABLES

Page

1. INTRODUCTION

1.1 Pathology and impact of laminitis

Laminitis is a common equine disease, having troubled horse owners for centuries with mention in literature dating back to ancient Greece [1]. The disease is widespread and devastating, frequently resulting in permanent lameness or euthanasia. It develops when the laminar tissue (Figure 1.1), a complex structure of overlapping epidermal and dermal laminae connecting the hoof wall and third phalanx, becomes inflamed. This inflammation can be precipitated by a variety of factors discussed later. In the acute stage of the disease, laminitis can be recognized by a bounding digital pulse at the fetlock and abnormally high hoof temperature [2]. Subsequent damage to the laminar tissue can lead to displacement of the third phalanx and transition into the chronic form of the disease. This acute to chronic transition is very common with up to 75 % of horses affected by acute laminitis developing chronic laminitis [3]. Given the frequency of occurrence of the disease and likelihood of long-term medical treatment or euthanasia, its economic impact is substantial. One USDA study found 13 % of equine farms surveyed were impacted by laminitis and 2.1 % of included horses were affected at some point in the one year period in which the study took place [4]. Another study estimated that 15 % of horses develop laminitis at some point in their lives, and that $13 million is lost annually in association with the disease due to mortality and medical expenses [3]. The pervasiveness and typically poor outcome associated with the disease make laminitis an important subject for further study. The condition is not restricted to domestic animals; a recent study found chronic laminitis affecting between 40 and 93 % of individuals sampled from five wild herds of Australian feral horses [5]. Despite

the prevalence and long reported history of the disease, owners of affected animals are left with few prevention and treatment options, particularly because the underlying causes of laminitis are not well understood. While there remains significant debate over what specific mechanisms lead to the development of laminitis (potential reasons outlined below), evidence has emerged pointing to a systemic inflammatory response in horses that develop the disease [6-8].

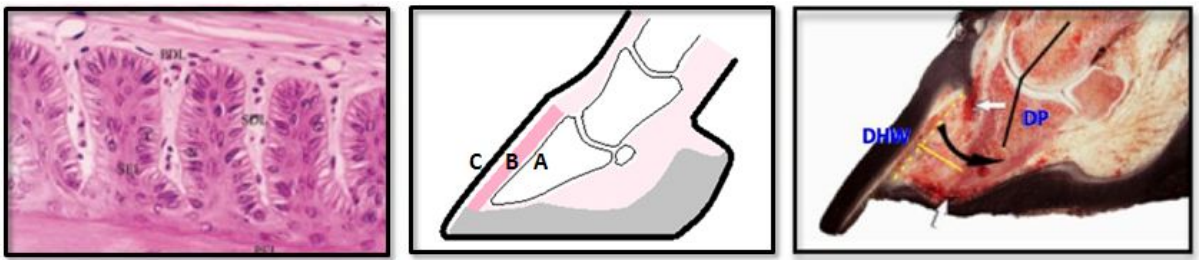


Figure 1.1 – Laminar tissue morphology. Left: a slice of laminar tissue stained with hematoxylin-eosin showing the primary dermal lamellae (PDL), secondary dermal lamellae (SDL), primary epidermal lamellae (PEL), and secondary epidermal lamellae (SEL) [9]. Middle: cross-section of the hoof showing the orientation of (A) the third phalanx, (B) the laminar tissue, and (C) the hoof wall. Right: rotation of the distal phalanx (DP) from its original position along the dotted yellow line and formation of the lamellar wedge (solid yellow line) in a horse with chronic laminitis [10].

A wide range of events can trigger the development of laminitis. Perhaps the best known of these is carbohydrate overload in which a horse develops laminitis subsequent to ingesting large quantities of carbohydrate rich food such as when a horse is given access to a lush pasture or abnormally large quantity of grain [4, 11, 12]. Laminitis has also been associated with trauma to the foot [13, 14], sepsis [15, 16], and metabolic syndrome [8, 17, 18]. While a variety of events precipitating laminitis have been identified, the mechanisms by which laminar tissue inflammation is initiated remains unknown. Changes in hindgut

microbiota have been implicated in the development of laminitis in a number of studies [19-22]. During oligofructose overload induced laminitis the population of bacteria has been shown to shift from primarily gram-negative to primarily gram-positive strains, and an increase in streptococcal species has been reported [19-21]. In the carbohydrate model streptococcal species were shown to decrease at 8 hours when compared with controls but to be similar to control values by 24 hours, and anaerobic streptococcal species were decreased at both time points [20]. It has been suggested that endotoxemia resulting from a shift in hindgut bacterial communities is responsible for inducing laminitis, although administration of lipopolysaccharide (LPS) does not itself induce laminitis [23]. Increases in both plasma LPS concentration [24] and serum immunoglobulin titers against core LPS [25] have been observed to increase in response to oligofructose induced laminitis and colic-causing gastrointestinal disorders, respectively. It has also been suggested that vasoactive amines may act to induce laminitis by causing ischemia and oxidative damage [26-28] and that matrix metalloproteinases (MMPs) [29, 30] and lactic acidosis resulting from bacterial shifts in the gut [31, 32] may be responsible for the degradation of the laminar tissue. There is also a metabolic component to laminitis, and horses with metabolic syndrome are predisposed to developing laminitis [8, 17, 18]. The mechanisms behind this endocrinopathic associated laminitis are not well understood, but it is thought that insulin resistance plays a role, and laminitis has been induced in live animals subjected to hyperinsulinemia [33, 34].

Although there are differences in the progression of laminitis brought on by the various causes of the disease, the basic course of the disease follows a common path. Laminitis can be characterized as acute in the initial 72 hours before permanent physiological changes to the foot have taken place, or chronic, after separation of the distal phalanx from

the hoof wall has occurred. The acute stage of laminitis is characterized by a bounding digital pulse, increased temperature in the hoof, and the onset of lameness. Lameness is often scaled according to the internationally accepted Obel Scoring System of 1-4 [35]. In the Obel Scoring System clinical signs range from Grade 1, with the horse frequently shifting weight and having a shortened stride at the trot, to Grade 4, with the horse exhibiting extreme reluctance to move. Increased infiltration of leukocytes into the laminar tissue can be measured within the first 24 hours of the disease. This has been observed in three models of laminitis: black walnut heartwood extract [6, 36], carbohydrate overload [37], and oligofructose administration [38] although this change was not seen following the administration of LPS [36].

Structural changes in the hoof also play an important role in the pathology of laminitis. The laminar tissue is an intricate structure, composed of overlapping dermal and epidermal laminae that supports the load-bearing distal phalanx within the hoof (Figure 1.1). The primary epidermal laminae (PEL) are an extension of the keratin-based hoof wall towards the interior of the hoof comprised of keratinocytes. The PEL attach to the secondary epithelial laminae (SEL), which are composed of a basal cells and extend in the direction of the distal phalanx [39]. The SEL are attached to the secondary dermal laminae (SDL) by the basement membrane, the extracellular matrix forming a structural partition between the dermis and epidermis composed of proteins including collagen and laminin [39-42]. Unlike the epidermal laminae the dermal laminae include vasculature, and consist of strong collagen-based connective tissue that is attached to the distal phalanx [39]. During laminitis this delicate architecture becomes disrupted and its ability to support the distal phalanx is greatly impaired, often leading to failure of the laminar tissue.

One of the earliest structural changes to occur during acute laminitis is the detachment of the basement membrane. Detachment has been measured at 48 hours post administration of carbohydrate [43] and 12 hours post oligofructose administration [38]. As the disease progresses, lesions are formed in the lamellae and the architecture of the tissue is compromised [33, 34, 43]. Additional damage occurs in the vasculature, and increased blood flow has been implicated as having a role in the failure of the laminar tissue [44]. Frequently, damage to the laminar tissue is so severe that the coffin bone becomes detached from the hoof wall, at which point the disease enters its chronic phase with associated life-long medical problems.

Chronic laminitis is characterized by rotation of the distal phalanx and the widening of the laminar region in the formation of the lamellar wedge (Figure 1.1) [10]. Studies on chronic laminitis have rarely been carried out, but those that have been conducted have found significant changes in the foot of affected individuals. Horses with chronic laminitis have substantial alterations in the structure of the basement membrane zone [45], the area separating the dermal and epidermal laminae, as well as significantly increased levels of bacteria isolated from the laminae when compared with controls (especially members in the phylum *Actinobacteria* and in the *Staphylococcus* genus) [46]. Carter et. al. [47] found that the regulation of epidermal stem cells is altered in horses with chronic laminitis. P63, a marker of proliferation potential for epidermal cells, is expressed at significantly lower levels in the hooves of horses with chronic laminitis and the authors propose that this change in expression has a causative role in the formation of the lamellar wedge. In addition to alterations in the basement membrane and the formation of the lamellar wedge, a neuropathic component has also been described in association with chronic laminitis. Expression of ATF,

a neuronal injury marker, and neuropeptide Y are increased in horses with chronic laminitis, in addition to changes in axon shape and reduction of fibers, and alteration in the myelin sheath in the lateral digital nerve [48]. Given that most horses who survive the acute stage of laminitis go on to develop chronic laminitis, further research on this stage of the disease is essential.

Changes in inflammatory cytokine expression in laminar tissue have been observed in various models of acute laminitis. The expression of IL-6 mRNA is upregulated in the black walnut extract [49, 50], carbohydrate [51] and oligofructose models [38]. The expression of IL-1β mRNA has also been shown to increase across all three models [49-52]. A number of other cytokines have altered mRNA expression profiles in the laminar tissue during acute laminitis including increases in IL-8 in the black walnut extract, oligofructose [49], and carbohydrate [50] models, and IL-12 in the carbohydrate model [51]. Tumor necrosis factor alpha (TNF-α) is a cytokine frequently elevated in inflammatory responses but showed no change in either the carbohydrate overload model [51] or the black walnut extract model of laminitis [50]. Other cytokines have shown inconsistent expression patterns between models or studies. One study found IL-2 and Interferon gamma (IFN-γ) expression to be increased in the oligofructose but not black walnut extract model [49]. Another study found no change in IFN-γ in carbohydrate induced laminitis [51]. The anti-inflammatory cytokine IL-10 was found to decrease during the onset stage of the black walnut extract model but not in later stages of the disease [49] and showed no change in two additional studies using the black walnut extract model [50] and carbohydrate overload model [51]. Interestingly, a study looking at the protein expression of inflammatory cytokines in which a prolonged euglycaemic hyperinsulinaemic clamp was used to induce laminitis [53] found that IL-6

protein expression was not increased in the laminar tissue but that there was an increase in the expression of TNF-α protein, in contrast to the previously described mRNA results for these cytokines. However, the study found no change in the expression of either protein when a prolonged glucose infusion was used as an alternate method of inducing laminitis.

1.2 Experimental models for laminitis

Previous studies on laminitis have predominantly relied on inducing the disease in live animals. Several disease models have been used, including carbohydrate or oligofructose overload and the administration of lipopolysaccharide or black walnut extract. While these studies have undoubtedly increased our understanding of the progression of the disease they are limited by several important disadvantages. The obvious drawback to this method is the need to euthanize often-healthy animals to complete a study, but there are also the associated problems of low sample numbers and high relative variability between animals. Additionally, all of these methods are based on inducing a systemic response with the effect of a subsequent inflammatory response in the laminar tissue. While this systemic model has its own merits, it has the disadvantage that the disease-causing bioactive compounds leading to inflammation in the laminar tissue may be many steps removed from the initial system insult. This disconnect can make it difficult to elucidate the mechanisms involved in initiating local laminar tissue inflammation. Thus, there is a need for a disease model that allows direct manipulation of the laminar tissue. In order to address this gap in experimental techniques, we have developed an in vitro tissue culture model for laminitis that allows the study of the effects of specific bioactive agents on laminar tissue inflammation. We use slices of tissue, which are more suitable as a model for laminitis than cell culture as the 3D architecture of

the laminar tissue, cell-cell contact, and extracellular matrix proteins are maintained. Tissue is cultured in Leibovitz's L-15 medium to mimic the non-proliferative conditions of laminar tissue in vivo. Many samples can be taken from one animal, both increasing replicate number and allowing comparison of treatments within an animal. In this way the effects of variability between animals on experimental results can be reduced. Additionally, our model can make use of tissue from horses euthanized for reasons unrelated to research. This model has the potential to reduce the overall cost and the number of horses used in trials if implemented in future laminitis studies.

While laminar tissue has been cultured in previous studies [38, 54], there have been significant shortcomings in replicating cellular processes in vivo. In one case [38], laminar tissue explants were large (15mm thick), which could potentially compromise cell survival in the center of the tissue. This study also used culture conditions that promoted cellular proliferation, which does not mimic non-proliferative in vivo cellular activity in the laminar tissue. A second study cultured thin slices (5mm x 5mm x1mm) in an assay measuring glucose uptake in the laminar tissue [54], also under culture conditions promoting cellular proliferation. An in vitro tissue culture method more accurately reflecting the cellular processes of the laminar tissue in vivo is an important need in laminitis research.

1.3 The role of apolipoprotein A-IV in laminitis

Despite the high frequency with which acute laminitis develops into the chronic form of the disease, few studies have focused on chronic laminitis and comparatively little is known about the initiation and progression of the disease at the molecular level. Chronic laminitis is associated with systemic changes in the horse, and Wagner et. al. [55] report that

horses with chronic laminitis have an increased response to intradermally administered allergens when compared with control horses but little is known about how these changes affect an animal's susceptibly to laminar tissue inflammation. A recent proteomics study [56] measured increases in proteins involved in coagulation, and clotting processes, providing additional support for a systemic shift in horses with chronic laminitis. Notably, this study also showed a greater than two-fold increase in plasma levels of apolipoprotein A-IV (apoA-IV) in horses with chronic laminitis when compared with controls [56]. In this study, differential gel electrophoresis (DIGE) was used to identify proteins with different concentrations in the plasma of horses with chronic laminitis than in controls. Of the 16 proteins having different plasma concentrations between the two groups, apoA-IV is of particular interest due to previous research indicating a novel role for this protein in mitigating inflammation [57].

Apolipoproteins are proteins that bind to lipids in the formation of lipoproteins, structures that allow for lipid transport in the circulatory and lymphatic systems. ApoA-IV was first described in 1977 in the high density lipoprotein fraction of rat serum [58] and later identified in the lymph of dogs and humans [59]. In humans and rats apoA-IV has a molecular weight of 46 kDa, and it has molecular weight of 44 to 45 kDa in dogs. In horses, apoA-IV has a unique size and expression profile which may be indicative of a unique function in this animal. Equine apoA-IV is reported to be around 30kDa [56], which is significantly smaller than reported in other species. Additionally, the predicted protein sequence from the NCBI protein sequence database [60] (reference sequence: XP_001502503.1) yields a predicted protein of 43.3kDa in the ExPASy tool ProtParam [61] indicating that the protein may be post translationally modified in horses.

In rats apoA-IV is synthesized in both the liver and the intestine, while in humans synthesis is limited to the intestine [62, 63]. Our lab has found apoA-IV to be expressed in the liver, white blood cells, and laminar tissue in horses (unpublished data). Further study into the structure and distribution of apoA-IV in horses may shed light on its function in horses and help to explain its association with chronic laminitis.

In addition to its role in lipid transport, a number of novel functions have been reported for apoA-IV. The protein plays a role in the short-term regulation of food intake [63] with hypothalamic apoA-IV levels becoming depleted during periods of food deprivation and increasing after feeding. ApoA-IV also increases insulin secretion in pancreatic islets under in vitro conditions of high glucose [64], is involved in the clearance of amyloid-β peptide in the brain [65], the accumulation of which is associated with Alzheimer's disease, and acts as an anti-oxidant [66].

Importantly, apoA-IV has an anti-inflammatory function in mice and is associated with chronic inflammatory diseases in humans. ApoA-IV knockout mice exhibit a more severe inflammatory response in dextran sulfate sodium induced colitis as compared to wild type mice, an effect that is substantially reversed through the administration of recombinant human apoA-IV [57]. These results demonstrate that apoA-IV has the ability to mitigate intestinal inflammation, and although the mechanism is not completely understood, the authors suggest that apoA-IV reduces leukocyte recruitment by inhibiting the expression of P-selectin in endothelial cells. Intriguingly, the plasma levels of ApoA-IV are increased in patients with rheumatoid arthritis [67] and ankylosing spondylitis [68], both conditions which involve chronic systemic inflammation. Although there is evidence linking increased apoA-IV levels and chronic laminitis, and the protein has been shown to have an anti-inflammatory

effect in mice, the effects of the protein on laminar tissue are unknown. Thus, further research is warranted to determine if apoA-IV plays a role in modulating inflammation in laminar tissue.

2. JUSTIFICATION FOR UNDERTAKING THE PROJECT

Equine laminitis is debilitating disease frequently occurring in horses, which has a significant impact on the equine industry. Laminitis is characterized by inflammation of the laminae, the supporting structure connecting the hoof wall to the third phalanx. The condition can be precipitated by a variety of factors including carbohydrate overload [4, 11, 12], insulin-intolerance [69], and mechanical damage [13, 14, 70]. Despite the high prevalence of the disease [4] and its typically poor outcome, little is known about the mechanisms driving laminar tissue inflammation and few treatment options are available. Currently, nearly all laminitis studies are conducted in live horses, a process that is both expensive and limited in biological replicates. A significant limitation in laminitis research today is the lack of a model system in which laminar tissue can be manipulated in vitro. Thus, the development of an in-vitro model for the disease is an important step in furthering laminitis research. To address this gap, in this study we characterize a model for inducing inflammation in slices of laminar tissue in culture. Laminar tissue was cultured in 1mm thick slices to ensure that the structure of overlapping dermal and epidermal laminae remains intact. Tissue was cultured in Leibovitz's medium to account for the non-proliferative conditions found in vivo. We tested two agents for inducing inflammation: Interleukin 6 (IL-6) and lipopolysaccharide (LPS) over a series of time points (4, 15, 24, and 48 h). In order to assess inflammation we measured IL-1β, IL-6, IL-8, TNF-α, and IFN-γ, cytokines that have been measured in previous in vivo laminitis studies [24, 37, 38, 49-52]. We also measured the expression of ADAMTS1, CCL2, CD14, S100A8, DEFB4, SOD2, and CXCL14, genes that have previously been shown to be differentially expressed in the oligofructose and carbohydrate

models of induced laminitis [71]. In the future this model could be used in elucidating the mechanisms behind laminar tissue inflammation and eventually in the development and testing of potential treatments for laminitis.

A recent study has identified several proteins that are upregulated in horses with chronic laminitis, notably apolipoprotein A-IV (apoA-IV) [56]. ApoA-IV has been shown to be correlated with chronic inflammatory diseases in humans [67, 68] and to have anti-inflammatory functions in mitigating experimentally induced colitis in mice [57]. These previous findings suggest that apoA-IV may be involved in the inflammatory response of horses experiencing laminitis. In this study we seek to determine whether apoA-IV plays a role in the development of laminar tissue inflammation through the use on an in-vitro model. Equine apoA-IV must have a different sequence from human apoA-IV because of its significantly smaller size, and its expression in white blood cells and laminar tissue is unique from the expression profile in humans and rodents [62, 63]. Determining how the sequence of equine apoA-IV varies from other species and the cellular source in the horse will provide important information about what type of functions it performs in this species. Despite the correlation between increased levels of apoA-IV and laminitis it remains unknown how the protein affects the laminar tissue, information that is key to understanding why it is increased in horses with laminitis. In order to address these gaps in knowledge, we planned to isolate and sequence apoA-IV, determine its full expression profile in the horse, and conducted a series of laminar tissue culture experiments in which we measured the effects of apoA-IV on inflammation in laminar tissue *in vitro*.

3. OBJECTIVES AND POTENTIAL OUTCOMES

The first objective of this project was to develop and characterize an in-vitro tissue culture model for equine laminitis. Our goal was to develop a tissue culture model for laminitis using 1 mm thick laminar tissue slices of laminar tissue cultured under non-proliferative conditions. The model was optimized to ensure that cells throughout the slice would survive and that the model remained contamination free. Two different agents, IL-6 and LPS, were used to induce inflammation in the tissue and the gene expression of inflammatory cytokines were measured by real time PCR to assess the degree of laminar tissue inflammation. Furthermore, gene expression in the model was compared with gene expression of laminitis associated genes identified in two in-vivo models for laminitis.

We expected that we would be able to develop a functional tissue culture technique in which the cells throughout the entire slice remain alive for 48 h without contamination. Although this method includes significant modifications to previously published techniques, we had already demonstrated the ability to keep laminar tissue viable over a period of at least 4 days as assessed by of an MTT assay. Thus, we anticipated that a viable model could be produced from this work. We had received on average approximately one horse per month from the Anatomic Pathology Laboratory at the Texas A&M University College of Veterinary Medicine and so anticipated that we will be able to access tissue from enough horses to further optimize the model and perform experiments on at least 5 animals.

The second objective of this project was to evaluate the role of apoA-IV in laminar tissue inflammation. Using the above model developed in our lab, our second goal was to measure both the effects of apoA-IV on inflammatory markers and the effects of induced inflammation on apoA-IV gene and protein expression. We also attempted isolate equine apoA-IV to determine the protein sequence through mass spectrometry. The sequence was to be compared with the human apoA-IV sequence in order to identify structural differences and determine if the smaller equine protein is missing any regions that are likely to be functionally important.

We expected that this research would result in a comprehensive profile of apoA-IV mRNA and protein expression in the horse given that we had already been able to measure APOA4 expression [72] and protein concentration [56] in our laboratory. We expected to be able to isolate high-purity apoA-IV for sequencing by following a protocol that has been successfully used previously to isolate bovine apoA-IV [73] and to find significant structural differences between equine apoA-IV and apoA-IV from humans because of the significant size difference already measured [56]. While we acknowledge that there are most likely multiple proteins important in the inflammatory response in laminitis, apoA-IV was an ideal candidate due to its known association with the disease [56] and its ability to mitigate inflammation [57]. We expected to measure a significant decrease in the concentration of inflammatory markers with the addition of apoA-IV based on the anti-inflammatory action previously reported for the protein [57]. Given that we have been successful in measuring differences in gene expression in laminar tissue culture we are confident we will be able to detect any differences between treatments.

4. MATERIALS AND METHODS

4.1 Tissue collection for APOA4 expression panel

Tissue samples were collected from 5 horses euthanized for reasons unrelated to this study, at the Anatomic Pathology Laboratory at the Texas A&M College of Veterinary Medicine. Only horses with a postmortem interval of less than 1 h were included. Samples from the small intestine, colon, cecum, liver, brain, stomach, tongue, pancreas, lung, heart, spleen, adipose tissue, and eye (Table 4.1) were collected in RNAlater ® (Qiagen, Valencia, CA) and stored on ice for transport to the laboratory where they were stored at 4˚C overnight, then frozen at -80˚ C after excess RNAlater ® was removed. As evident from Table 4.1, only 5 tissue samples could be obtained from all animals.

Tissue	**Horse**			
	1	**2**	**3**	**4**
Adipose		x		
Brain	x	x	x	x
Cecum	x	x	x	x
Colon	x	x	x	x
Eye	x			
Heart	x	x		
Liver	x	x	x	x
Lung	x	x		
Pancreas	x	x		
Skeletal muscle	x	x		
Small intestine	x	x	x	x
Spleen		x	x	
Stomach		x	x	
Tongue	x	x		

Table 4.1 – Tissue collected from each of 5 horses. An x marks that the tissue was collected from that individual.

4.2 Laminar tissue collection and culture

Laminar tissue from 5 separate horses from those used in the tissue panel was obtained from the Anatomic Pathology Laboratory at the Texas A&M College of Veterinary Medicine. Only horses euthanized for reasons unrelated to lameness arising from the hoof with a postmortem interval of less than 1 h were used. Tissue was collected from the two front feet of each horse. Laminar tissue was dissected from along the sagittal plane of the hoof after it was transected with a band saw (Figure 4.1). Tissue was then sectioned into 5 x 5 x 1 mm slices using a McIlwain Tissue Chopper (Mickle Laboratory Engineering Co. Ltd., Surrey, UK) and washed 3 times in PBS containing the fungicide amphotericin B (2.5µg/ml; Life Technologies, Grand Island, NY). Two fresh slices were collected and frozen at -80˚C (one snap frozen in liquid nitrogen for protein analysis and one first stored overnight at in RNAlater ® at 4˚C for RNA analysis). The remaining slices were cultured in a 48-well plate at 37˚C (Figure 4.1). Each slice was cultured in 300µl Leibovitz's media with amphotericin B (0.5µg/ml; Life Technologies) and penicillin-streptomycin (100u/ml penicillin and 100ug/ml streptomycin; Life Technologies).

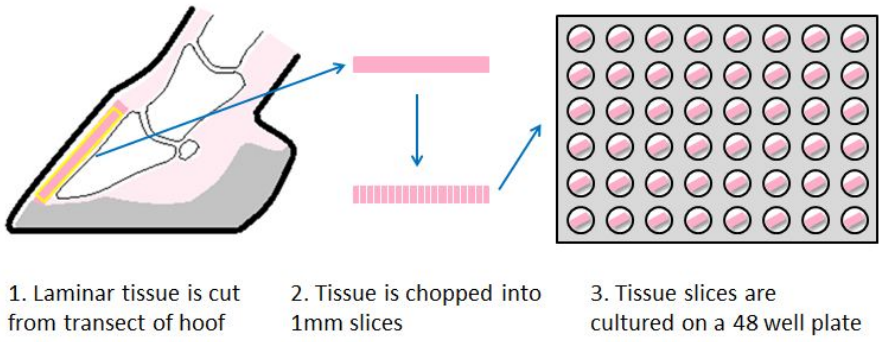


Figure 4.1 – Steps in tissue collection and culture preparation.

Tissue from each horse was subjected to the following treatments: a control sample in media alone, BSA (as a control for the samples containing IL-6, which required BSA as a carrier), LPS (an inflammatory agent), IL-6 (a second inflammatory agent), endotoxin-free recombinant human apoA-IV (to test the effects of the protein alone), apoA-IV and LPS (to test the effects of apoA-IV on the development of LPS-induced inflammation), and apoA-IV and IL-6 (to test the effects of apoA-IV on the development of IL-6-induced inflammation) (Table 4.2). Two slices from each treatment were collected at each time point (4, 15, 24, and 48 h): one for measurement of protein concentration by western blot (snap frozen in liquid nitrogen) and one for measurement of gene expression (stored at 4˚C overnight in RNAlater ® then frozen at -80˚C).

Treatment concentrations	**Sources of reagents**
Control (Leibovitz's media only)	Life Technologies (Grand Island, NY)
BSA (10μg/ml)	Sigma-Aldrich, Co. (St Louis, MO)
Recombinant human apoA-IV (25μg/ml)	Novoprotein (Summit, NJ)
Recombinant equine IL-6 (100ng/ml)	R&D Systems, Inc. (Minneapolis, MN)
LPS (1μg/ml)	Sigma-Aldrich, Co. (St. Louis, MO)
IL-6 (5ng/ml) + apoA-IV (25μg/ml)	See above
LPS (1μg/ml) + apoA-IV (25μg/ml)	See above

Table 4.2 – Tissue culture treatments.

Four additional slices were cultured in media alone to verify cell survival by staining with MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) (Life Technologies). MTT is a light-yellow tetrazolium dye that is reduced to dark purple formazan by dehydrogenase enzymes in metabolically active cells, and can be used to verify cell survival. After the addition of MTT (0.25μg/ml) to culture wells, the plate was placed

back in the incubator at 37˚C for 30 min, after which qualitative visual confirmation of cell viability throughout the slice was conducted.

4.3 RNA isolation

Individual tissue slices were minced finely with a scalpel and homogenized with a hand-held pellet pestle motor in 1ml QIAzol Lysis reagent (Qiagen). RNA was isolated according to the manufacturer's protocol. RNA was treated with DNase (RNase-free set, Qiagen), followed by a cleanup with the RNeasy Mini Kit (Qiagen) according to the manufacturer's instructions. RNA concentration was determined by spectrophotometer (Nanodrop, Germany).

4.4 Reverse transcription

Samples were checked for genomic DNA contamination prior to reverse transcription by running a PCR reaction for the gamma actin gene (see Table 4.3 for primer sequences). The reaction included 5 ng/µl RNA, 0.025 units/µl JumpStart^{TM} REDTaq® DNA polymerase (Sigma-Aldrich, Co., St Louis, MO), 200µM dNTPs (Life Technologies) and 0.6µM gamma actin forward and reverse primers (Sigma-Aldrich) in 1X PCR Buffer (Sigma-Aldrich). Reactions were run by heating samples to 95°C for 1 min followed by 31 cycles of: 94°C (30 sec), 58°C (30 sec), 72°C (30 sec), then cooled at 72°C for 5 min before being held at 12°C. The PCR product was run on a 2% agarose gel containing 0.01µg/ml ethidium bromide at 100v for 1 h and imaged using a BioDoc-It ® Imaging System (UVP, LLC, Upland, CA). Samples showing no DNA contamination were reverse transcribed using the Superscript VILO cDNA Synthesis Kit (Life Technologies) according to manufacturer instructions.

Thereafter, 250ng RNA was added to each reaction (the maximum amount possible for the least concentrated RNA samples). Reactions were run with the program: 25°C for 10 min, 42°C for 1 h, 85°C for 5 min, and then held at 12°C. Negative controls included reactions wherein the reverse transcriptase had been omitted.

GENE	FORWARD (5'-3')	REVERSE (5'-3')
ACTG	CAGGATGCAGAAGGAAATCA	CGCAGTCCATTTAGAAGCAT
EEF1A1	TGGAAAGAAGCTGGAAGATG	CAACCGTCTGTCTCATGTCA
APOA4	GCCCGGGCTGAGGTCAATGC	AAAGCGTGTTGAGCTTCTGGGTGA
IL-1β	CCAGAGGCGGCCGGGACATAAC	GGGAAGGCAGCTGGGCATTGATT
IL-6	ACTCCTTCTTCACAAGCACCGTC	AGTGGGGTGGGGAAAGCAGTAG
IL-8	GAGTGGGCCACACTGCGAAA	TGGATTTTGCCCCTCAGCTCTCTT
TNF-α	CTGGCCCAGACACTCAGATCATCT	CATTTGCACGCCCACTCAGC
IFN-γ	TACCTATTACTGCCAGGCCGCG	ATCCAGGAAAAGAGGCCCACCA
ADAMTS1	AGGCTCACAATGAATTTTCG	CACAGCCAGCTTTTACACACT
CCL2	CAGAAACCAACAACTCTCAGG	TATAGCAGCAGGTGACTGGAG
CD14	CAGCTCTTTCCAGAGTCCAC	AGTTCTCATCGTCCACCTCA
CXCL14	ATCACCACCAAGAGTGTGTCC	TAT GCCTGCGAGAAAGAAAG
DEFB4	TTCCTCATTGTCTTCCTGTTG	ACTTAGGGGATATGCAGAAGC
S100A8	ACGGATCTGGAGAATGCTATC	TGATGTCCAACTCTTTGAACC
SOD2	ACTTTGGTTCCTTCGACAAAT	CAGGGGAATAAGACCTGTTGT

Table 4.3 – Primer sequences. Housekeeping genes (red), APOA4 (yellow), inflammatory cytokine genes (green) and genes up-regulated in laminar tissue across 3 models of induced laminitis (blue).

4.5 Qualitative PCR measurement of APOA4

APOA4 expression was measured using the PCR conditions described above (see Table 4.3 for primer sequences). Each reaction included 2µl of cDNA (corresponding to 25ng of RNA added to the reverse transcriptase reaction). The PCR product was run on a 2% agarose gel containing 0.01µg/ml ethidium bromide at 100v for 1 h and imaged using a BioDoc-It ® Imaging System (UVP, LLC, Upland, CA).

4.6 Measurement of APOA4 expression in tissue panel by qRT-PCR

RNA was isolated and reverse transcribed as described above. Gene expression was measured by qPCR (see Table 4.3 for primer sequences) on a LightCycler 480 machine (Roche Diagnostics Co., Indianapolis, IN). Reactions were run with 1µl of cDNA in 20ul of 1x SYBR Green I Master mix (Roche Diagnostics Co.) containing 0.5µM primers. Reactions were first denatured at 95°C for 5 min, then cycled through: 95°C (10 sec), 58°C (5 sec), and 72°C (10 sec) 45 times. Negative controls included reactions without the addition of template cDNA. Data analysis was performed using the Pfaffl method [74]. Samples were normalized to gamma actin and calculated as a fold change from the average across all tissues.

4.7 Measurement of gene expression from tissue culture experiments by qRT-PCR

Gene expression of 5 inflammatory cytokines (IL-1β, IL-6, IL-8, TNF-α and IFN-γ) and 7 genes found to be increased during laminitis across multiple models in a previous study (ADAMTS1, CCL2, CD14, CXCL14, DEFB4, SOD2 and S100A8) (Table 4.4) [71], were measured by qRT-PCR (see Table 4.3 for primer sequences). RNA was isolated from one slice of tissue at each time point as described above, and reverse transcribed as described above with reactions including 600 ng RNA. qRT-PCR was conducted as described above with 30 ng cDNA per reaction. Data analysis was performed using the Pfaffl method [74] with gamma actin as a control gene. EEF1A1 was measured as a second control gene in the laminitis-associated genes, and gamma actin was chosen for use over the entire experiment due to its lower variability across time and treatment. The effect of treatment in the tissue culture assays was assessed by two-way ANOVA using Prism (GraphPad Inc., La Jolla, CA). A p-value less than 0.05 was considered sufficient to reject the null hypothesis. Significant

differences between treatments were further analyzed to determine which time points were affected with a post-hoc Sidak's multiple comparisons test.

Gene	Protein	Function
IL1B	Interleukin-1 beta	Inflammatory response, apoptosis, differentiation, and cellular proliferation
IL6	Interleukin-6	Inflammatory response, B-cell maturation
IL8	Interleukin-8	Inflammatory response, angiogenic factor
TNF	Tumor necrosis factor alpha	Inflammatory response, coagulation, apopoptosis, cellular proliferation and differentiation
IFNG	Interferon gamma	Immunoregulation, macrophage activation, anti-viral and anti-tumor functions
ADAMTS1	A disintegrin and metalloproteinase with thrombospondin motifs 1	Acts to cleave aggrecan (a proteoglycan found in cartilage), and is associated with inflammation and weight loss in cancer
CCL2	Chemokine (C-C motif) ligand 2	Chemotactic signal for monocytes and basophils
CD14	Cluster of differentiation 14	Mediates immune response to LPS by binding its monomeric form and presenting it to the TLR4 complex
CXCL14	Chemokine (C-X-C motif) ligand 14	Inflammatory response and immunoregulation. Attracts neutrophils and dendritic cells.
DEFB4	Beta-defensin 2	Inflammatory regulation and antibiotic activities
SOD2	Superoxide dismutase 2, mitochondrial	Transforms toxic superoxide anion radicals into hydrogen peroxide and diatomic oxygen
S100A8	S100 calcium binding protein A8	Inflammatory response and immunoregulation. Complexes with S100A9 to form calprotectin. Involved in neutrophil recruitment and scavenging oxidants to reduce tissue damage.

Table 4.4 – Gene names and functions.

4.8 Sequencing of primer product

PCR amplification for each primer (Table 4.3) was run as described above in a 10µl reaction. The product was cleaned by adding 4µl ExoSap-it ® (Affymetrix, Santa Clara, CA) and incubated in a thermal cycler at 37°C for 15 min followed by 15 min at 80°C. A terminator reaction was then run using 1µl of cleaned-up PCR product, 2µl Big Dye v 1.1 (Life Technologies), 1µl of 2µM forward primer, and 1µl of sterile water. The reaction was run in a thermo cycler at 96°C for 1 min followed by 25 cycles of: 96°C for 10 sec, 50°C for

5 sec, and 60°C for 4 min, then held at 4°C. Excess dNTPs were removed with BioMax Spin-50 mini-columns (BioMax Inc., Arnold, MD). Columns were centrifuged at 1000 x g for 3 min at room temperature and flow-through discarded. Terminator reaction product was then added to the center of each column and spun for 3 min at 1000 x g, and the purified sample was recovered. Samples were lyophilized in a Vacufuge ® vacuum concentrator (Eppendorf, Hamburg, Germany), and submitted to the DNA Technologies Core Laboratory at Texas A&M University for sequencing with an ABI 3130xl Genetic Analyzer (Life Technologies). Sequences were edited by hand using Sequencher DNA Sequence Analysis Software (Gene Codes Corporation, Ann Arbor, MI). The resulting sequences were run through a nucleotide BLAST search [75] of the *Equus caballus* genome, restricted to highly similar sequences.

4.9 Protein isolation

Laminar tissue slices were finely minced with a scalpel and homogenized with a hand-held pellet pestle motor (Kimble Chase Kontes) using plastic DNase-free/RNase-free pestles in 900ml lysis buffer (0.02M Tris, 0.005M NaCl, 1% Triton-X 100). Buffer contained 1 cOmplete Mini EDTA-free Protease Inhibitor Cocktail Tablet (Roche Diagnostics Co.) per 10ml. Samples were shaken for 2 h on an ice block at room temperature followed by centrifugation at 12,000 rpm for 20 min at 4˚C. Protein-containing supernatant was removed and stored at -80˚C. Protein concentrations were measured using a BCA protein assay kit (Thermo Fisher Scientific Inc.) in which 25µl sample was added to 200µl working reagent and absorbance at 420nm was measured on a FLUOstar OPTIMA microplate reader (BMG Labtech, Ortenberg, Germany). Two replicates of each sample were averaged, and concentrations were calculated from a BSA standard curve.

4.10 2D-gel electrophoresis

2D gel electrophoresis was conducted at the Protein Chemistry Lab at Texas A&M University. Laminar tissue protein (70 μg) isolated as described above was added to each of three 13 centimeter immobilized 4-7 pH gradient IPG DryStrips (GE Healthcare, Fairfield, CT). Isoelectric focusing was conducted for 50,200 Vh at 50 μA. Proteins were then separated on three 12% polyacrylamide gel using the Hoefer^{TM} SE 600 Series Vertical Electrophoresis System (Fisher Scientific International Inc., Hampton, NH). Two gels were then transferred to a nitrocellulose membrane by electrophoresis at 30v for 1.5 h. One western blot was conducted using monoclonal anti-human apoA-IV antibody and a second was conducted using polyclonal anti-human apoA-IV antibody as described below. The third gel was stained for 1 h in GelCode Blue Safe Protein Stain (Thermo Fisher Scientific Inc.) and destained in water overnight. Spots that were stained by the western blot in the regions surrounding 28 kDa and 45 kDa were selected for mass spec analysis and the corresponding stained sections in the GelCode Blue stained gel were cut out and trypsin-digested for analysis at the San Antonio Proteomics Core service lab for LC-MS/MS analysis at the University of Texas.

4.11 Mass spec protein identification

X! Tandem (The GPM, thegpm.org) and Mascot (Matrix Science, London, UK) were used to analyze the samples. Identifications made by searching the NCBI database of non-redundant proteins were validated with the use of Scaffold 4 (Proteome Software Inc., Portland, Oregon).

4.12 Immunoprecipitation

Immunoprecipitation was conducted using the Crosslink Magnetic IP/Co-IP Kit (Thermo Fisher Scientific Inc.). Magnetic beads were washed 2x in coupling buffer, then bound to antibody in coupling buffer containing 0.05µg/µl anti-human apoA-IV (in ascites fluid) for 15 min at room temperature on a rotating platform. Plasma was used as a control for antibody in ascites fluid. Beads were washed 4x in coupling buffer, and incubated overnight at 4°C in undiluted plasma on a rotating platform. Beads were then washed 3x in wash buffer and 1x in water. IP product and antibody were eluted in 2 washes of 100µl elution buffer each lasting 5 min. Product from both washes was neutralized with provided neutralization buffer and combined. Immunoprecipitation product was precipitated by methanol-chloroform precipitation for detection of apoA-IV by western blot. First, 100µl product from the immunoprecipitation protocol was added to 400µl methanol in a 1.5ml Eppendorf tube. Samples were vortexed and 100µl chloroform was added followed by another vortex step and the addition of 300µl of water. Samples were then centrifuged at 13,000 x rpm for 1 min at room temperature and the upper layer removed. Next, 300µl water was added, samples were again vortexed, then centrifuged at 13,000 x rpm for 2 min at room temperature. Supernatant was removed and individual pellets were air-dried and dissolved in NuPAGE ® sample buffer containing DTT (dithiothreitol) as a reducing agent (Life Technologies) to be run on a gel for western blotting.

4.13 Plasma depletion

Equine plasma was processed with the Human IgY14 Spin Column Kit (Sigma-Aldrich, Co.) according to manufacturer instructions to deplete the top 14 most abundant

plasma proteins. IgY14 Spin Column was washed 2X with Dilution Buffer. Plasma was diluted (15µl into 485µl Dilution Buffer) and filtered through a Corning ® Costar ® Spin-X filter (Sigma-Aldrich) for 1 min at 10,000 x g. Filtered plasma was applied to the column and mixed for 15 min, then spun 30 sec at 2,000 rpm and flow-through collected. The column was then washed 3 times with 500µl Dilution Buffer Column and flow-through was saved and pooled with the first flow-through. The column was stripped 3 times for 5 min with Stripping Buffer and spun 30 sec at 2,000 rpm after each round, discarding flow through. The column was then neutralized with Neutralization Buffer for 5 min at room temperature, then spun for 30 sec at 2,000 rpm, and washed three times with Dilution buffer. Pooled flow-through was concentrated in an Amicon Ultra-15 10,000 NMWL Centrifugal Filter Unit (Millipore, Billerica, MA) to around 500µl. The concentrated flow-through was then re-applied to the column and the depletion steps repeated a second time.

4.14 Measurement of apoA-IV protein expression by western blot

Samples were prepared in NuPAGE ® sample buffer containing 50mM DTT as a reducing agent when run under reducing conditions (Life Technologies) and heated for 10 min at 70°C. When non-reducing conditions were used samples were prepared in NuPAGE ® sample buffer (Life Technologies) without reducing agent or heating step. Samples were run on pre-poured NuPAGE® Novex® 4-12% bis-tris polyacrylamide gels in NuPAGE® MOPS SDS running buffer (Life Technologies) for 45 min at 180v, and then transferred to nitrocellulose membrane for 90 min at 30v in NuPAGE® Transfer Buffer using the XCell SureLock^{TM} Mini-Cell Electrophoresis System (Life Technologies). To measure apoA-IV, the membrane was blocked with 5% milk in TBST (0.15M NaCl, 0.01M Tris, 0.1% Tween-

20) for 1 h at room temperature, incubated with monoclonal anti-human apoA-IV antibody (Abcam, Cambridge, UK) diluted 1:1000 in 5% milk overnight at 4˚C, washed 3x10 min, incubated with affinity purified polyclonal HRP-conjugated goat anti-mouse IgG diluted 1:5000 (Abcam) in 5% milk, and washed 3x15 min. Bands were visualized using the Immun-Star™ WesternC™ chemiluminescence kit (Bio-Rad Laboratories, Hercules, CA) according to manufacturer instructions. For western blots with HRP-conjugated VeriBlot anti-mouse antibody (Abcam) monoclonal anti-human apoA-IV was used as a primary antibody at a dilution of 1:1000 and HRP-conjugated VeriBlot anti-mouse antibody was used as a secondary antibody at a dilution of 1:1000. When testing HRP-conjugated goat anti-rabbit antibody (Abcam), a western blot was conducted as described above without a primary antibody (incubated overnight in 5% milk in TBST alone) and HRP-conjugated goat-anti rabbit as a secondary antibody at a concentration of 1:5000. A western blot with HRP-conjugated apoA-IV antibody (see below) was conducted as described above with HRP-conjugated antibody at 1:160. Following the first three washes after an overnight incubation in the antibody, an additional 15-min wash step was added, and the membrane was immediately visualized.

4.15 HRP-conjugation of apoA-IV antibody

Monoclonal anti-human apoA-IV (Abcam) was conjugated to HRP using the EasyLink HRP Conjugation Kit (Abcam). First, 5µl EL-modifier was added to 6µl antibody diluted into 45µl PBS and incubated for 3 h in the dark at room temperature. Then 5µl EL-Quencher reagent was added, and the HRP-conjugated antibody solution was stored in the dark at 4°C.

5. RESULTS

5.1 Tissue culture

Tissue culture viability: Tissue remained viable for the duration of the experiment as determined by staining with MTT. Two of 140 samples were contaminated as assessed by microscopic examination and were discarded. Several inflammatory cytokines and laminitis-associated genes were affected by the tissue culture alone, showing differences over time in the control and BSA control when compared back to the fresh tissue as assessed by ANOVA. Of the inflammatory cytokines (Figure 5.1), IL-1β varied significantly over the course of the experiment in both the control and BSA control treatments, and IL-8 varied significantly over time in the control treatment only. IL-6, TNF-α, and IFN-γ did not change over time in either treatment. Of the laminitis-associated genes (Figure 5.2), CXCL14 expression changed significantly over time in both the control and BSA control treatments while ADAMTS1 and DEFB4 expression were changed only in the BSA control treatment. SOD2 and S100A8 expression was altered significantly over the course of the experiment, but no specific differences between time points were identified by post hoc testing in either the control or BSA control treatment. The BSA control treatment was not significantly different from the control treatment for expression of any of the genes measured at any time point.

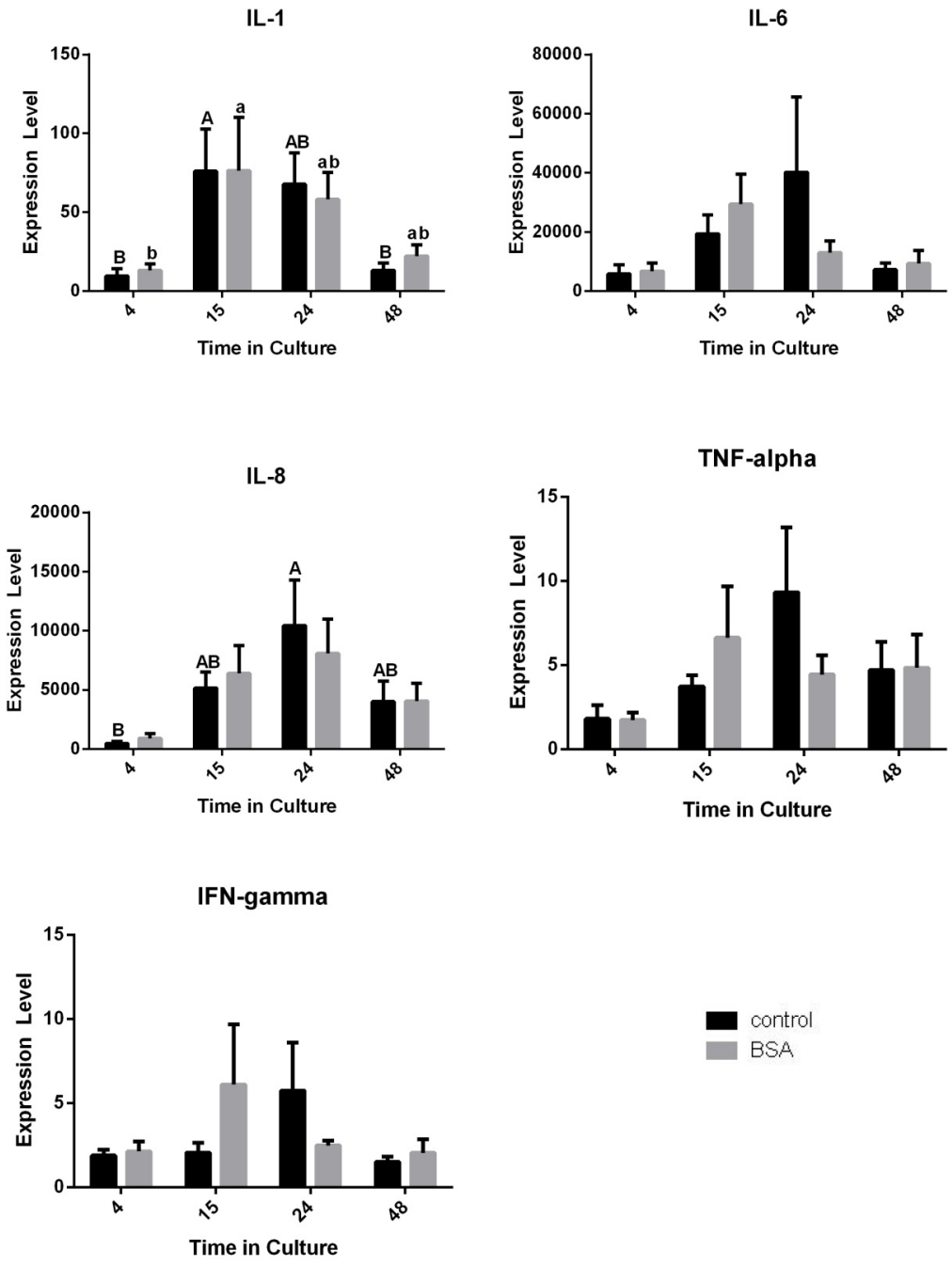


Figure 5.1 – Effects of culture over time on inflammatory cytokine gene expression for control and BSA control treatments compared back to fresh tissue. Significant differences ($p \leq 0.05$) are shown by non-overlapping uppercase (control) or lowercase (BSA control) letters. Data are presented as means ± SEM.

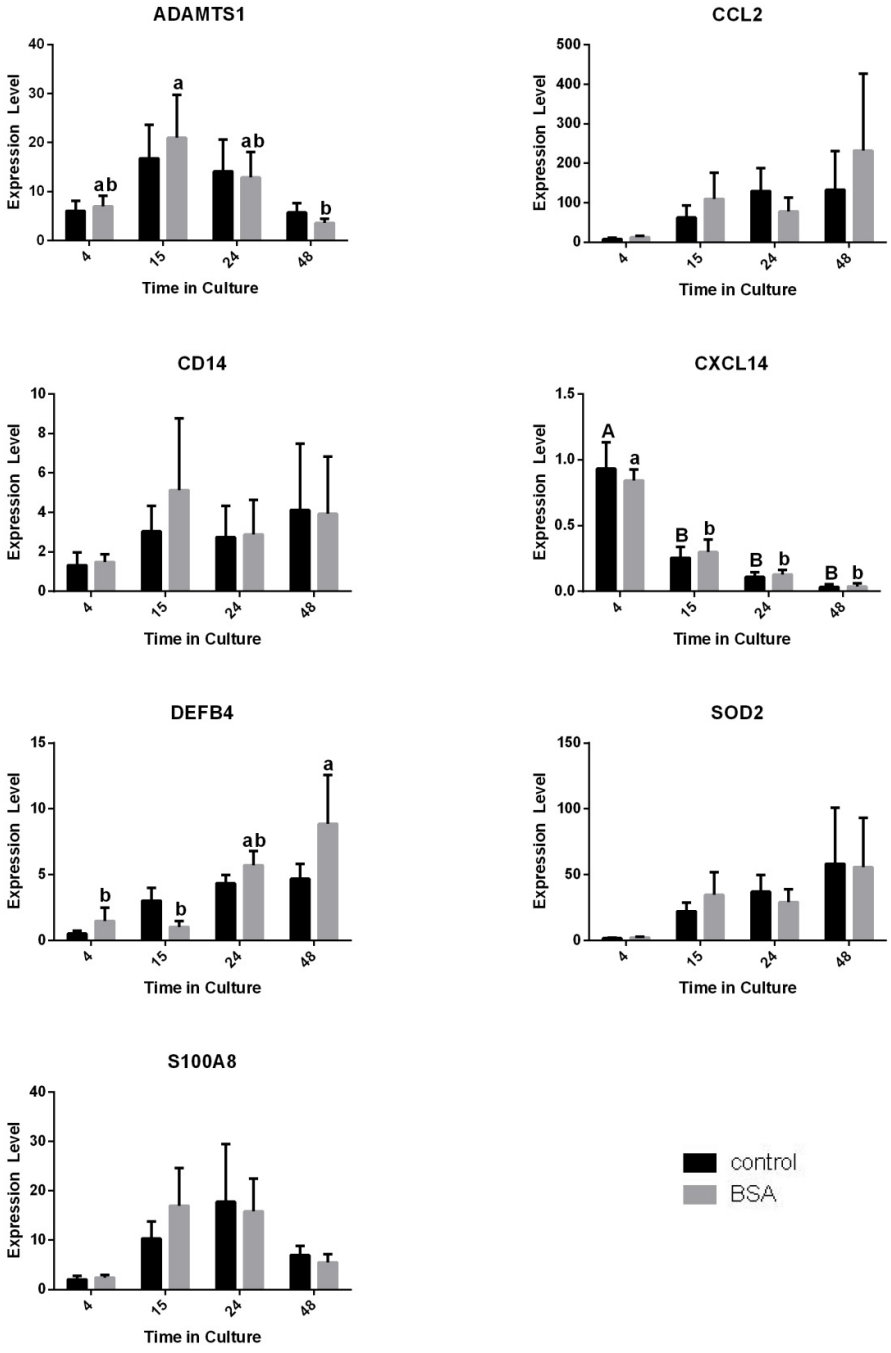


Figure 5.2 – Effects of culture over time on laminitis-associated gene expression for control and BSA control treatments compared back to fresh tissue. Significant differences ($p \leq 0.05$) are shown by non-overlapping uppercase (control) or lowercase (BSA control) letters. Data are presented as means ± SEM.

Primer product sequencing: All primers were validated through sequencing of amplified products to amplify the correct gene.

IL-6*:* IL-6 had no significant effect on the expression of any of the five inflammatory cytokines (IL-1β, IL-6, IL-8, TNF-α and IFN-γ) as assessed by ANOVA at any of the time points measured or over the course of the experiment as a whole when compared to the vehicle control (Figure 5.3). IL-6 also had no significant effect on any of the seven laminitis-associated genes (ADAMTS1, CCL2, CD14, CXCL14, DEFB4, S100A8 and SOD2) at any of the time points measured or over the course of the experiment as a whole when compared to the vehicle control (Figure 5.4).

LPS*:* LPS significantly increased the expression of IL-1β ($p \leq 0.001$), IL-6 ($p \leq 0.001$), and IL-8 ($p < 0.0001$) at 4 h as assessed by post hoc testing but did not significantly affect the expression of TNF-α or IFN-γ at any of the individual time points or over the course of the experiment as a whole relative to the control (Figure 5.5). LPS significantly increased the expression of CCL2 and SOD2 (Figure 5.6) over the course of the experiment ($p < 0.05$) as assessed by ANOVA, but no differences were found in their expression at any of the individual time points. There was no effect on the expression of ADAMTS1, CD14, CXCL14, DEFB4 or S100A8 (Figure 5.6) over the course of the experiment or at any individual time point.

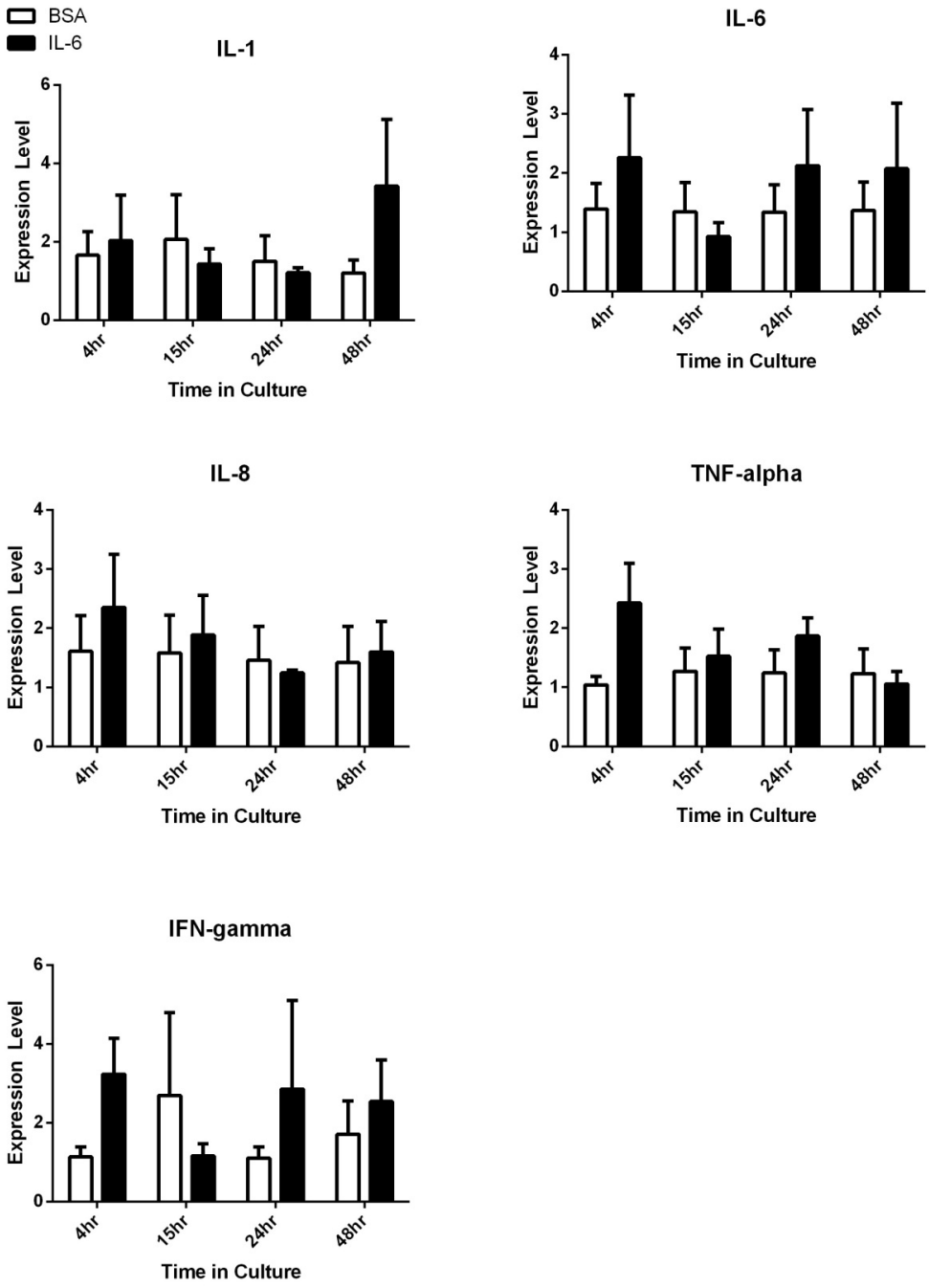


Figure 5.3 – The addition of IL-6 (100ng/ml) had no significant effect on the expression of any of the 5 inflammatory cytokines IL-1β, IL-6, IL-8, TNF-α or IFN-γ. Data are presented as means ± SEM.

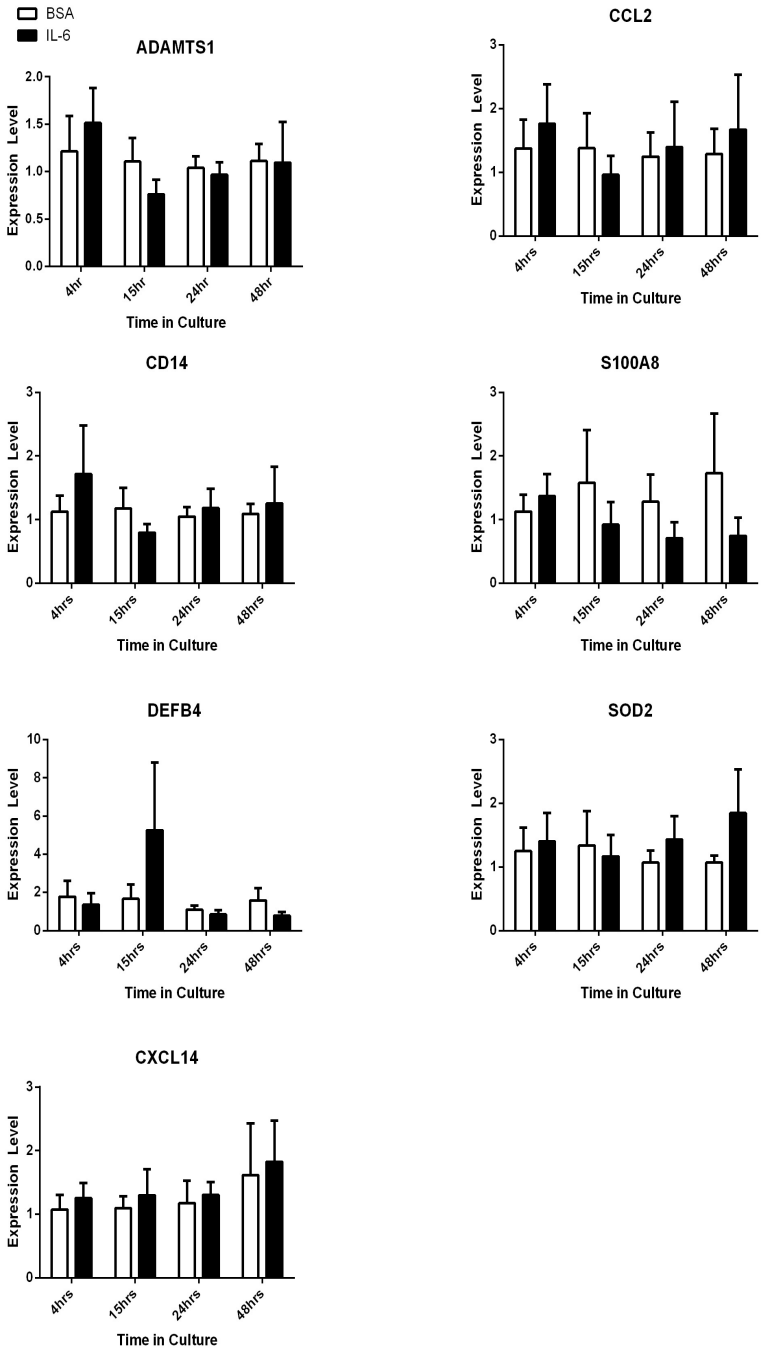


Figure 5.4 – The addition of IL-6 (100ng/ml) had no significant effect on the expression of any of the 7 laminitis-associated genes ADAMTS1, CCL2, CD14, CXCL14, DEFB4, SOD2 and S100A8. Data are presented as means ± SEM.

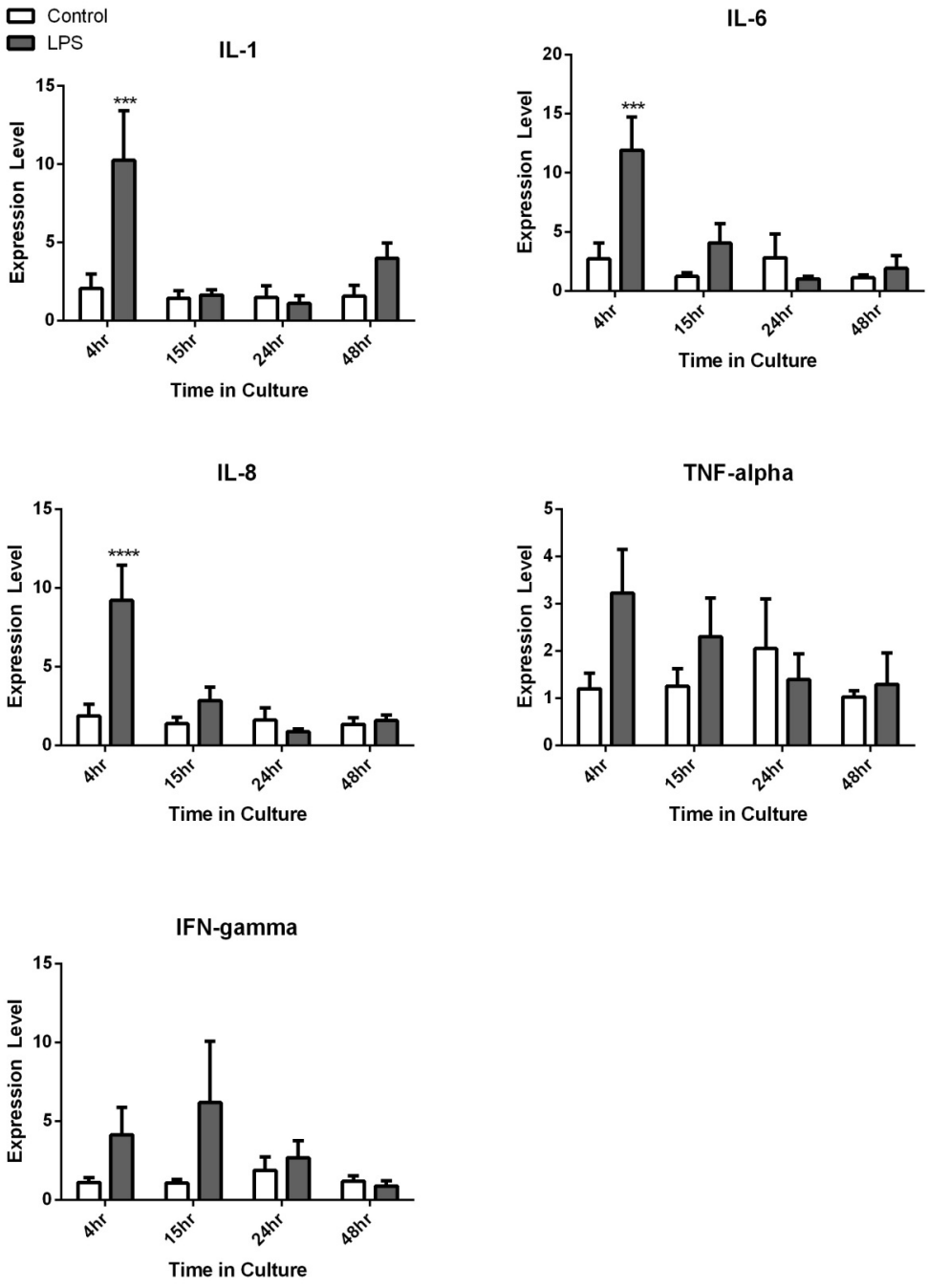


Figure 5.5 – The addition of LPS (1μg/ml) significantly increased the expression of IL-1β ($p \leq 0.001$), IL-6 ($p \leq 0.001$), and IL-8 ($p \leq 0.0001$) at 4 h but had no effect on the expression of TNF-α or IFN-γ at any time points. Data are presented as means ± SEM.

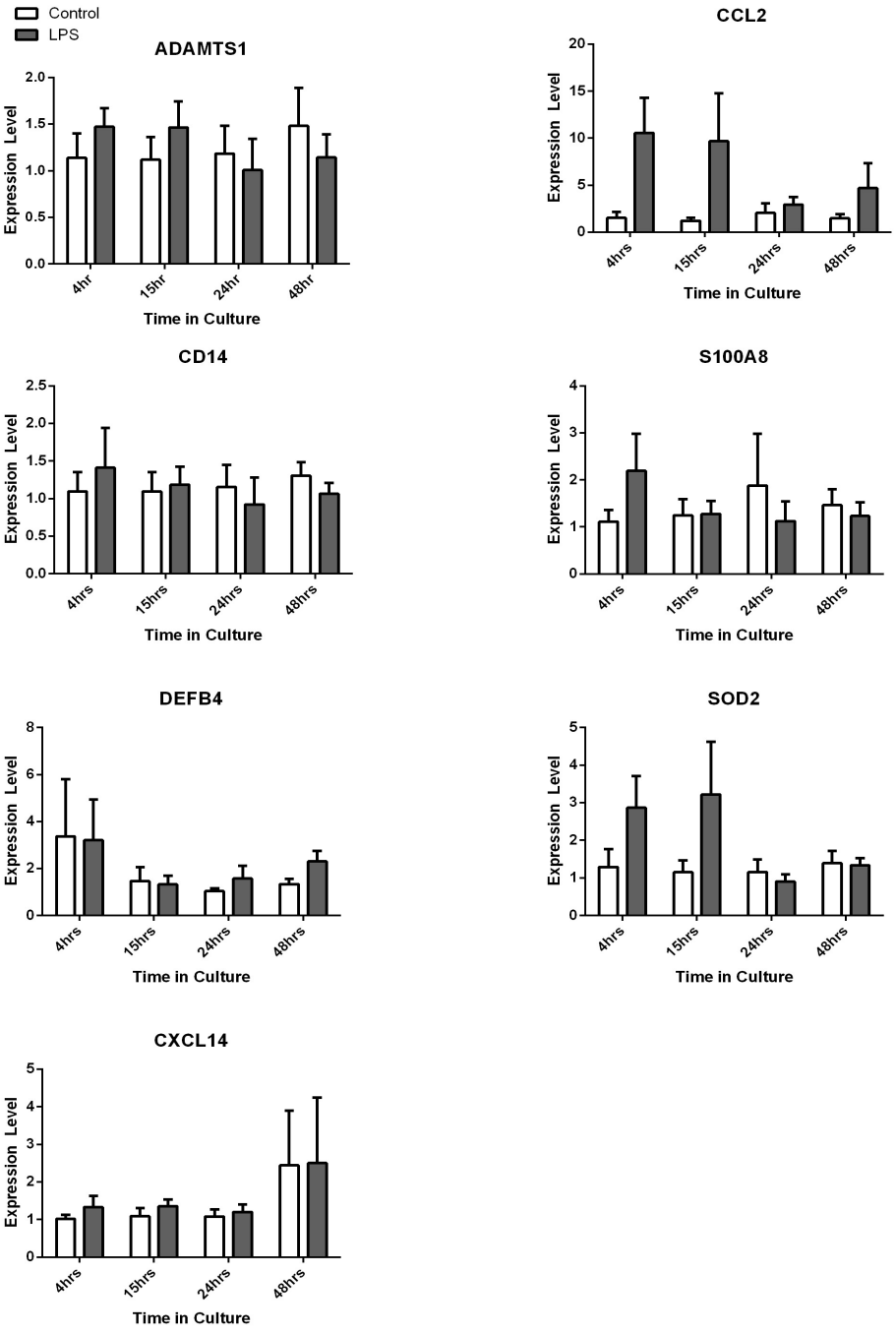


Figure 5.6 – The addition of LPS (1µg/ml) significantly increased the expression of CCL2 and SOD2 across the entire experiment ($p < 0.05$) but no differences were found at any individual time point. There were no significant differences in the expression of ADAMTS1, CD14, CXCL14, DEFB4 or S100A8 at any time point or over the course of the experiment. Data are presented as means ± SEM.

Cytokine correlations*:* To determine if the differences between horses in cytokine expression were robust, we looked at correlations between cytokine levels (Figure 5.7) at the 4 h time point, where significant differences between groups had been measured. In general, horse 2 and horse 5 showed lower correlation between cytokine expression levels than the other 3 animals. The cytokines that showed significant differences in expression at the 4 h time point in response to LPS (IL-1β, IL-6 and IL-8) were well correlated, with R^2 values averaging from 0.815 to 0.931 across the 5 animals. Correlations involving TNF-α and IFN-γ had low average R^2 values across the 5 animals, with none exceeding 0.711 (for TNF-α and IL-8), and 4 of the 7 relationships having R^2 values of below 0.4 (Table 5.1).

Inter-animal variation*:* Cytokine values were compared between horses in the LPS treatment group at the 4 h time point, the only time point where significant differences from the control were seen (Figure 5.8). Horses tended to be either high responders (horses 1, 3, and 4) or low responders (horses 2 and 5). In particular, horse 5 had lower expression levels than the average for all cytokines (Table 5.2), and the lowest expression of cytokines measured to be significantly different in response to LPS treatment (IL-1β, IL-6, and IL-8). Because horse 5 had consistently lower cytokine expression levels than the other animals in addition to relatively low cytokine correlations, we re-analyzed the data after the omission of horse 5. Removal of this animal, however, did not change the conclusions of our analysis.

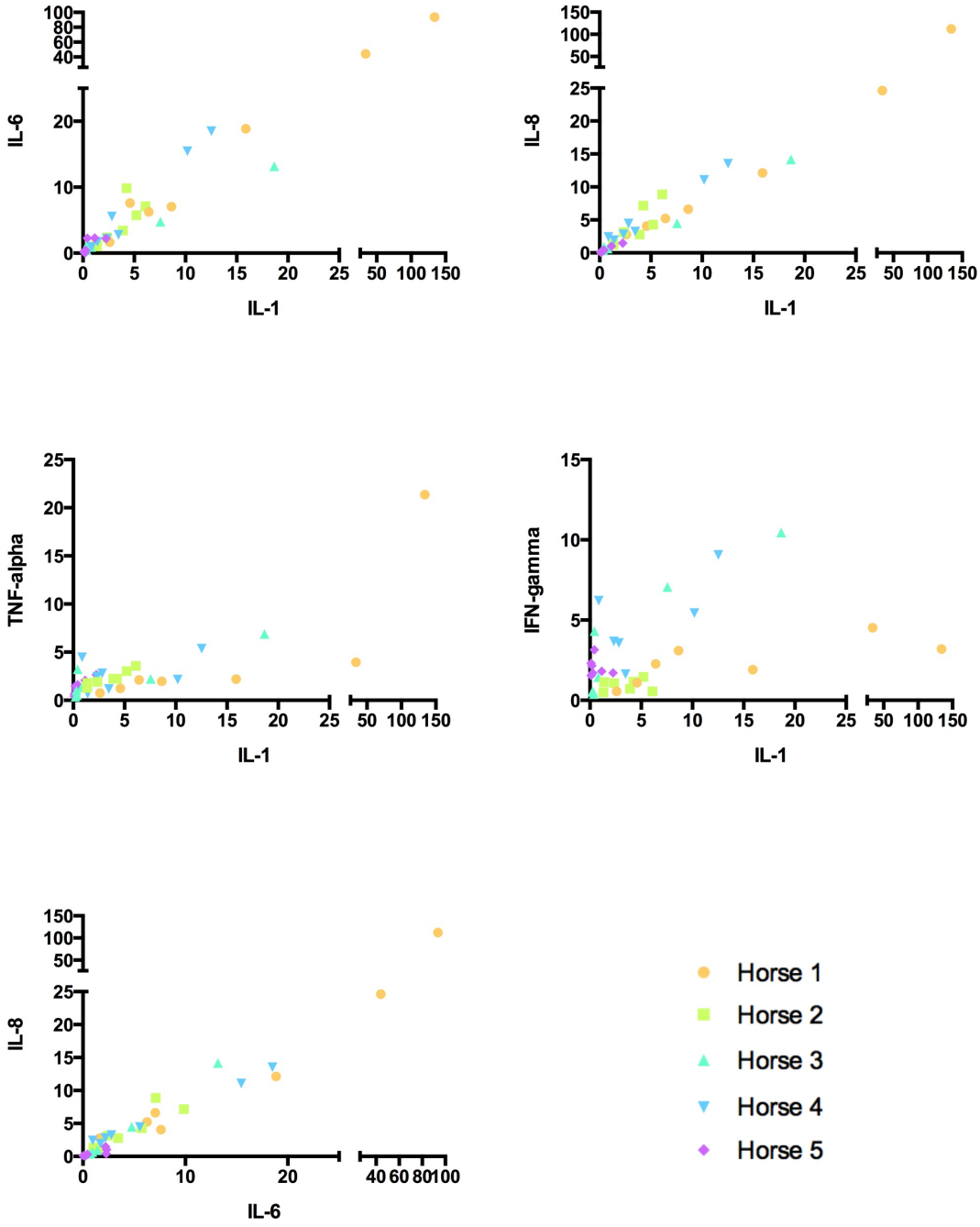


Figure 5.7 – Correlations between inflammatory cytokine expression levels at the 4 h time point in each of the 5 horses.

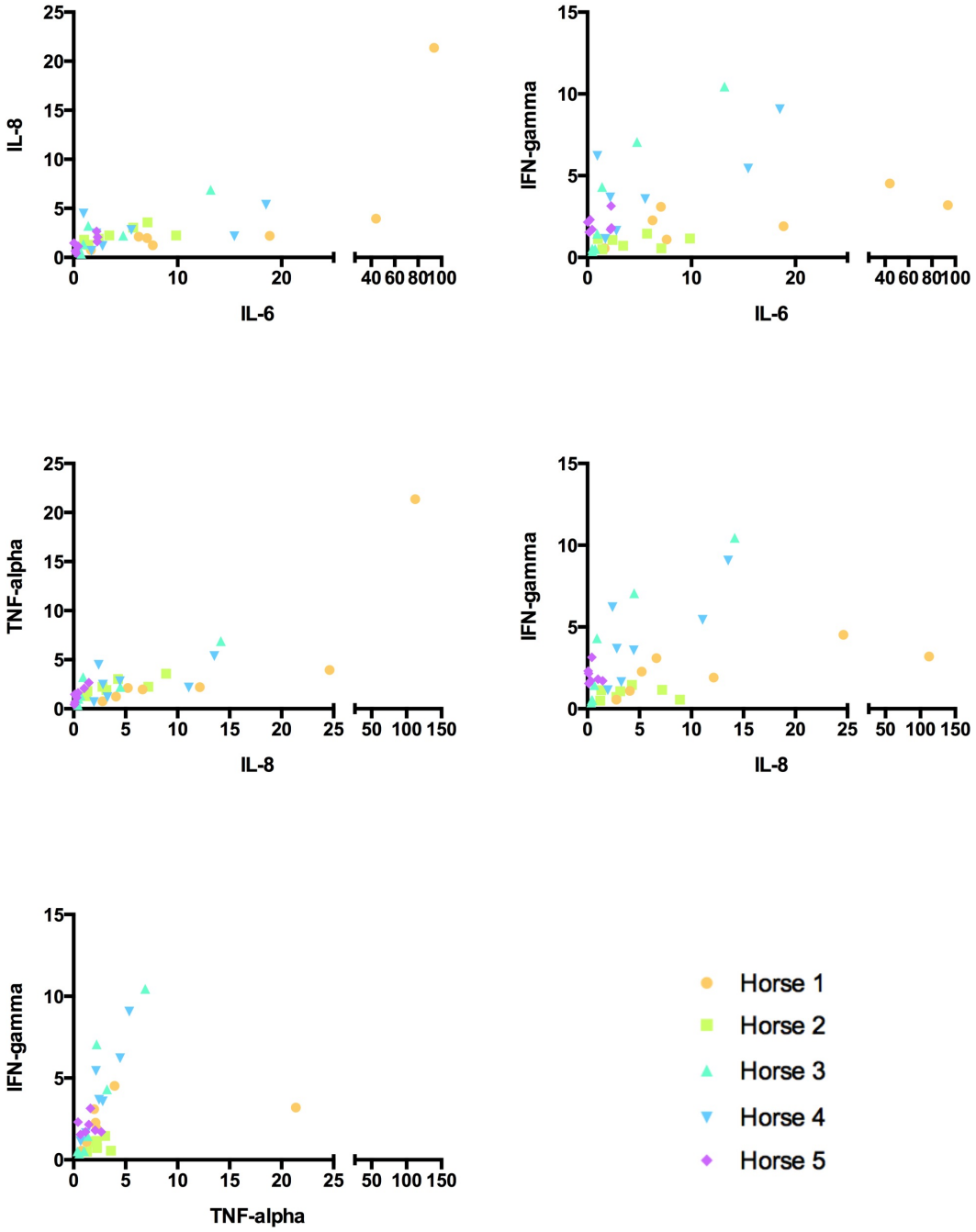


Figure 5.7 (continued)

Cytokine	R^2 Value					
	Horse 1	Horse 2	Horse 3	Horse 4	Horse 5	Average
IL-1β/IL-6	0.952	0.625	0.991	0.974	0.531	0.815
IL-1β/IL-8	0.999	0.721	0.989	0.981	0.964	0.931
IL-6/IL-8	0.935	0.798	0.994	0.998	0.655	0.876
IL-6/TNF-α	0.899	0.798	0.212	0.854	0.655	0.683
TNF-α/IL-1β	0.988	0.625	0.183	0.797	0.964	0.711
TNF-α/IL-8	0.994	0.630	0.258	0.846	0.819	0.710
TNF-α/IFN-γ	0.149	0.012	0.870	0.837	0.003	0.374
IFN-γ/IL-1β	0.195	0.008	0.476	0.847	0.073	0.320
IFN-γ/IL-6	0.325	0.045	0.509	0.851	0.072	0.360
IFN-γ/IL-8	0.170	0.001	0.568	0.820	0.065	0.325

Table 5.1 – R^2 values for correlations between inflammatory cytokine expression levels in all samples in each of the 5 horses.

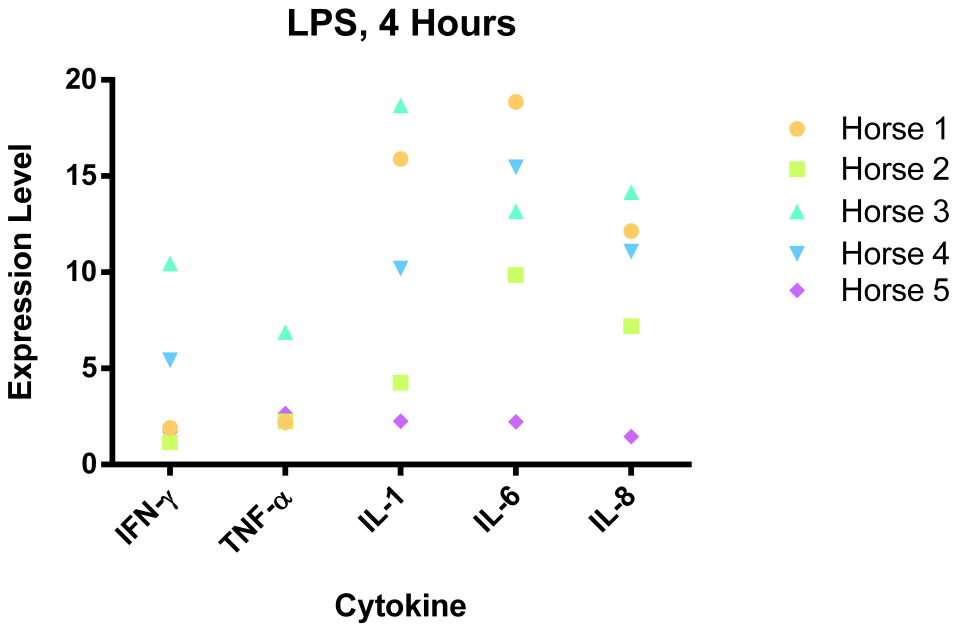


Figure 5.8 – Expression values of inflammatory cytokines in LPS treated samples after 4 h in each horse.

Cytokine	Expression Level					
	Horse 1	Horse 2	Horse 3	Horse 4	Horse 5	Average
IL-1β	15.88967	4.254074	18.67075	10.1872	2.253305	10.2509998
IL-6	18.85686	9.854927	13.17479	15.46233	2.217667	11.9133148
IL-8	12.13428	7.184288	14.1596	11.07846	1.462101	9.2037458
IFN-γ	1.902659	1.162189	10.45201	5.432763	1.711241	4.1321724
TNF-α	2.205364	2.234334	6.886201	2.150521	2.662982	3.2278804

Table 5.2 – Expression values for inflammatory cytokines in LPS treated samples after 4 h.

5.2 ApoA-IV

APOA4 expression panel*:* APOA4 was detected in at least one animal in the small intestine, colon, cecum, liver, brain, stomach, tongue, lung, heart, spleen, adipose tissue, and eye by qualitative PCR (Figure 5.9). APOA4 could also be detected in the same tissues by real-time PCR with the highest expression in the liver (Figure 5.10). Neither technique identified APOA4 as expressed in the pancreas of either of the two animals for which this organ was sampled. When the liver was excluded from the tissue culture panel, the highest expression was noted in the small intestine. Small intestine expression was higher than liver expression in the horse with the lowest liver expression of APOA4.

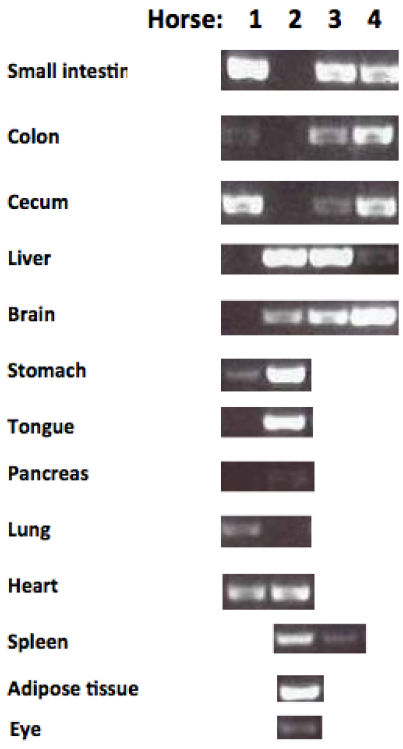


Figure 5.9 – Qualitative PCR results for tissue panel of APOA4 expression. 10µl PCR product was run on a 2% agarose gel.

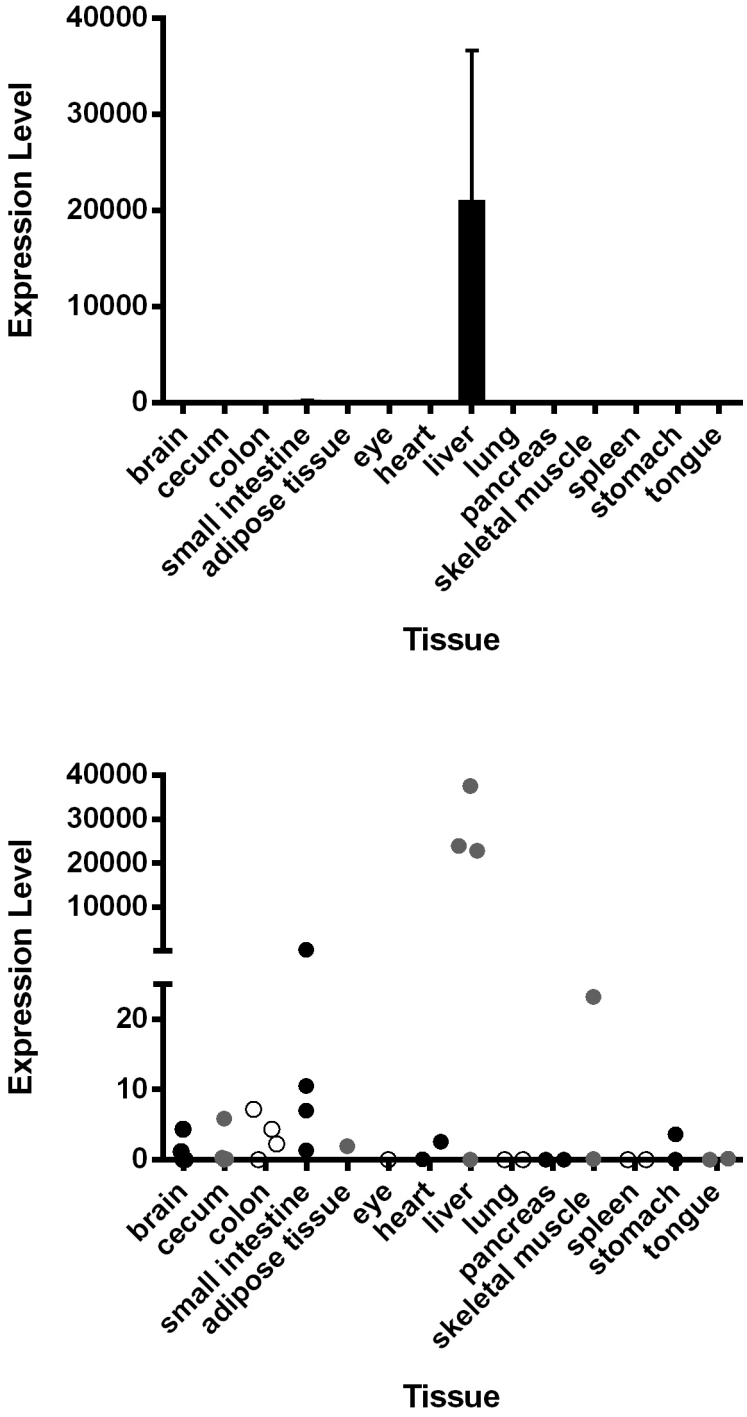


Figure 5.10 – Real-time PCR results for tissue panel of APOA4 expression. Expression value are normalized to gamma actin, and compared to the average expression across all tissues in the culture. Circles show expression level for individual horses in each tissue.

Addition of recombinant apoA-IV to tissue culture*:* The addition of recombinant human apoA-IV (25μg/ml) alone to cultured laminar tissue did not result in a significant change in expression for any of the inflammatory cytokines (Figure 5.11) or laminitis associated genes (Figure 5.12) as assessed by ANOVA. Because the highest levels of inflammatory cytokines and laminitis associated gene expression occurred at the 4 h time point, we compared apoA-IV treatment to the control group at the 4 h time point using a paired t-test, but found no significant differences. Although there appeared to be an increase in the expression of inflammatory cytokines and laminitis-associated genes (Figures 5.11 and 5.12), this was primarily due to a single animal (horse number 1). The addition of apoA-IV in combination with LPS (Figures 5.13 and 5.14) or with IL-6 (Figures 5.15 and 5.16) had no significant effect on gene expression. The results did not differ significantly from adding LPS or IL-6 alone in either case.

APOA4 expression in tissue culture*:* The expression of APOA4 was not significantly affected by the addition of LPS or IL-6, and was also unchanged by the addition of apoA-IV alongside LPS and IL-6 (Figure 5.17). However, expression of apoA-IV was very low in the laminar tissue, with an average Ct value of 35.86 (ranging from 29.69 to 40) and an average difference between replicates of 1.22, which likely contributed to the high standard error across samples for APOA4 expression.

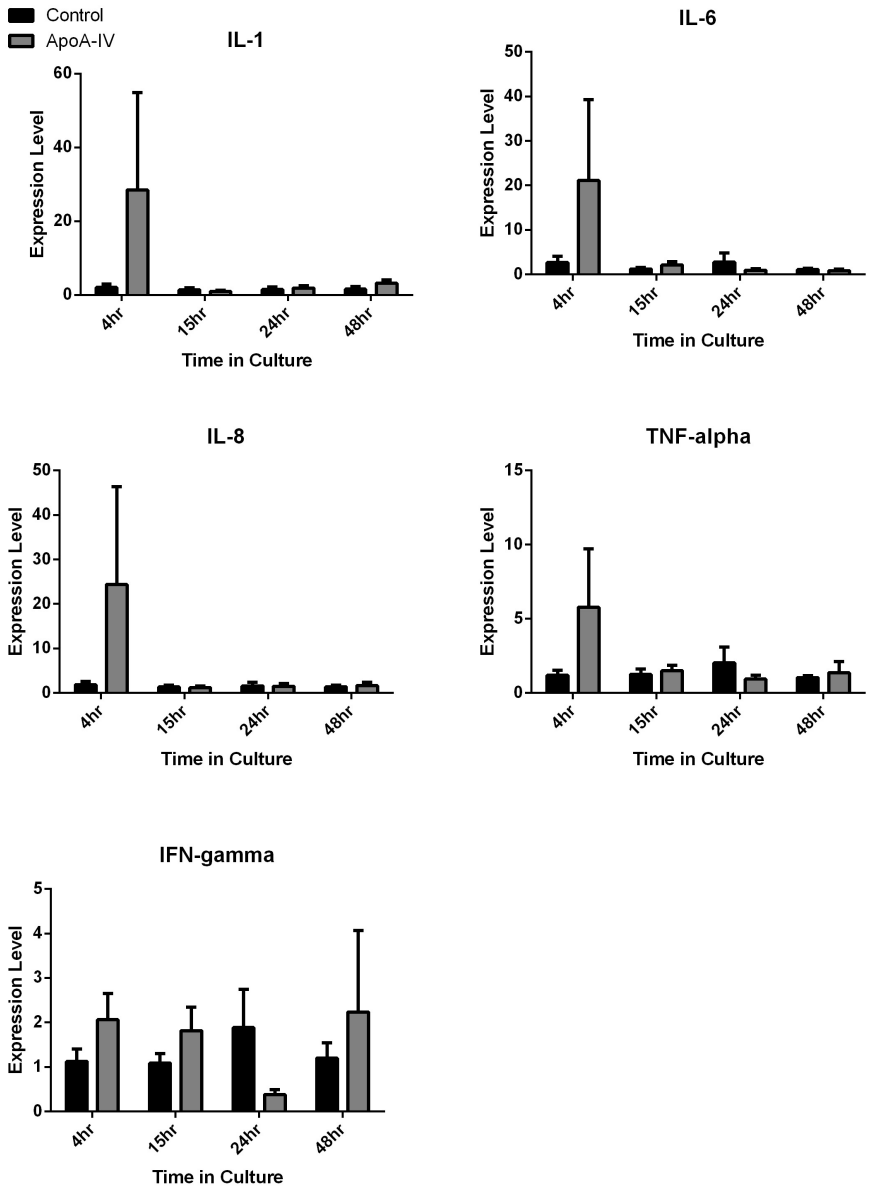


Figure 5.11 – The addition of recombinant human apoA-IV (25µg/ml) did not significantly affect the expression of inflammatory cytokines IL-1β, IL-6, IL-8, TNF-α or IFN-γ. Data are presented as means ± SEM.

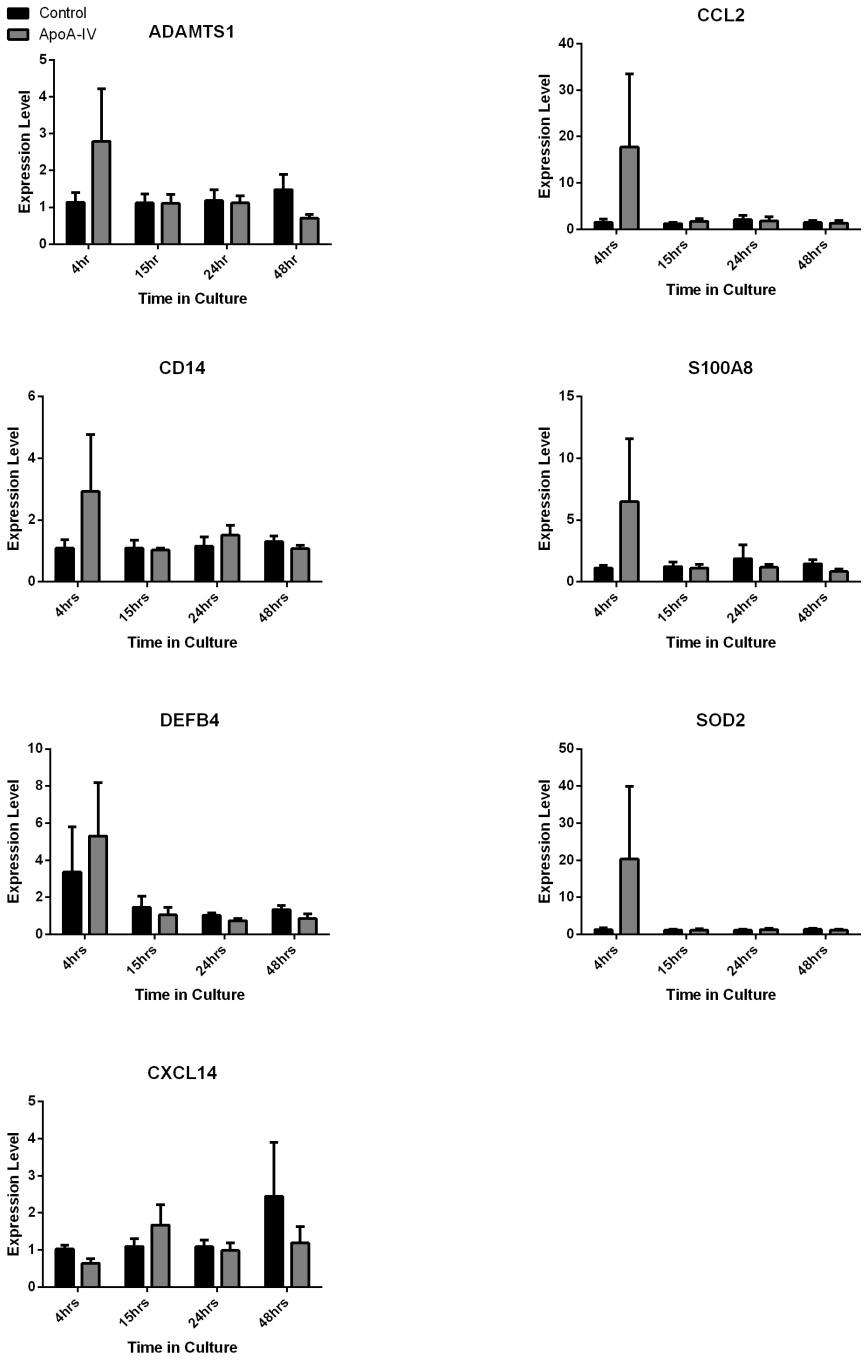


Figure 5.12 – The addition of recombinant human apoA-IV (25µg/ml) did not significantly affect the expression of laminitis-associated genes ADAMTS1, CCL2, CD14, CXCL14, DEFB4, S100A8 and SOD2. Data are presented as means ± SEM.

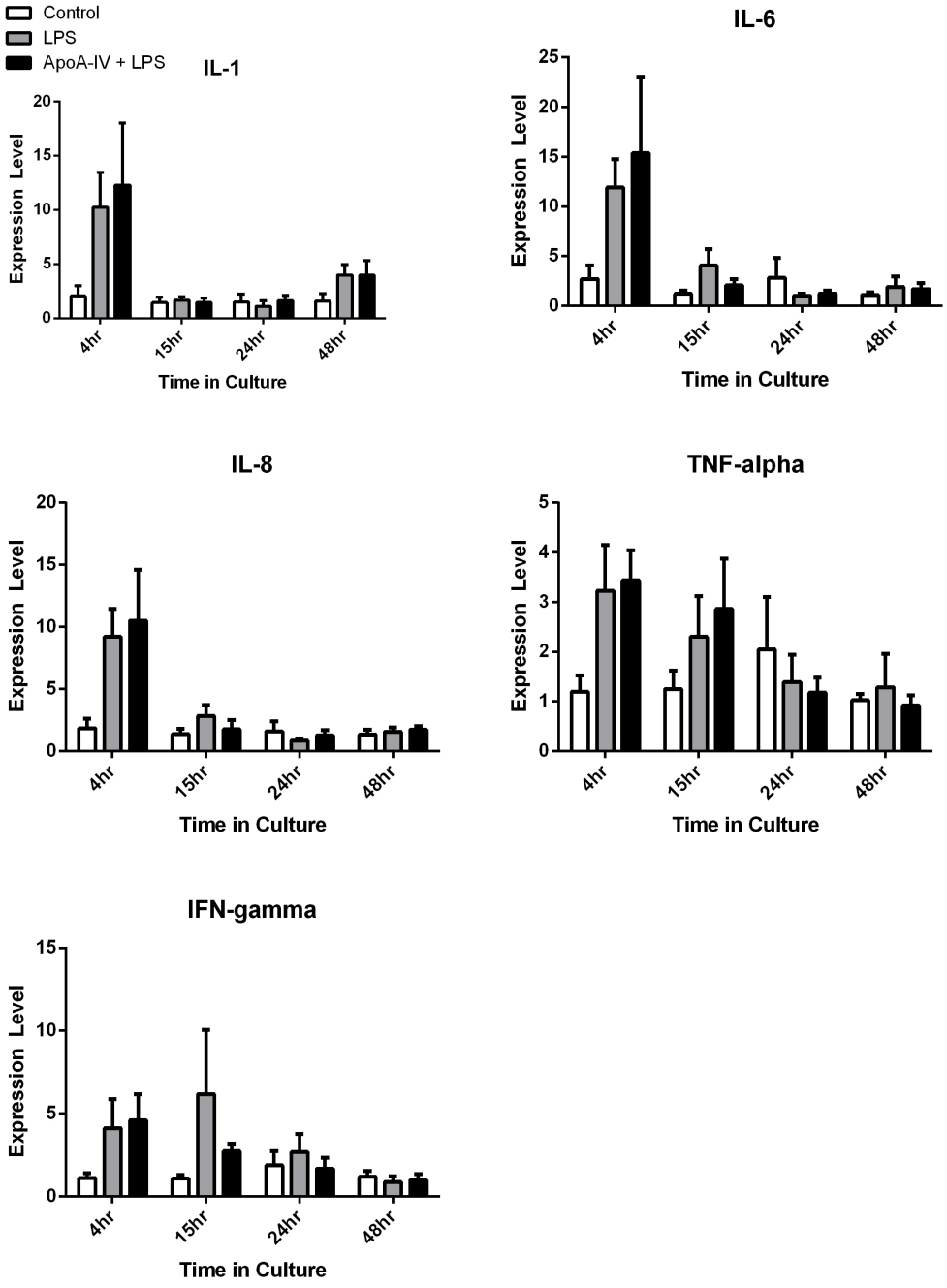


Figure 5.13 – The addition of recombinant human apoA-IV (25µg/ml) with LPS had no significant effect on the expression of the inflammatory cytokines (IL-1β, IL-6, IL-8, TNF-α and IFN-γ) when compared to the LPS treatment. Data are presented as means ± SEM.

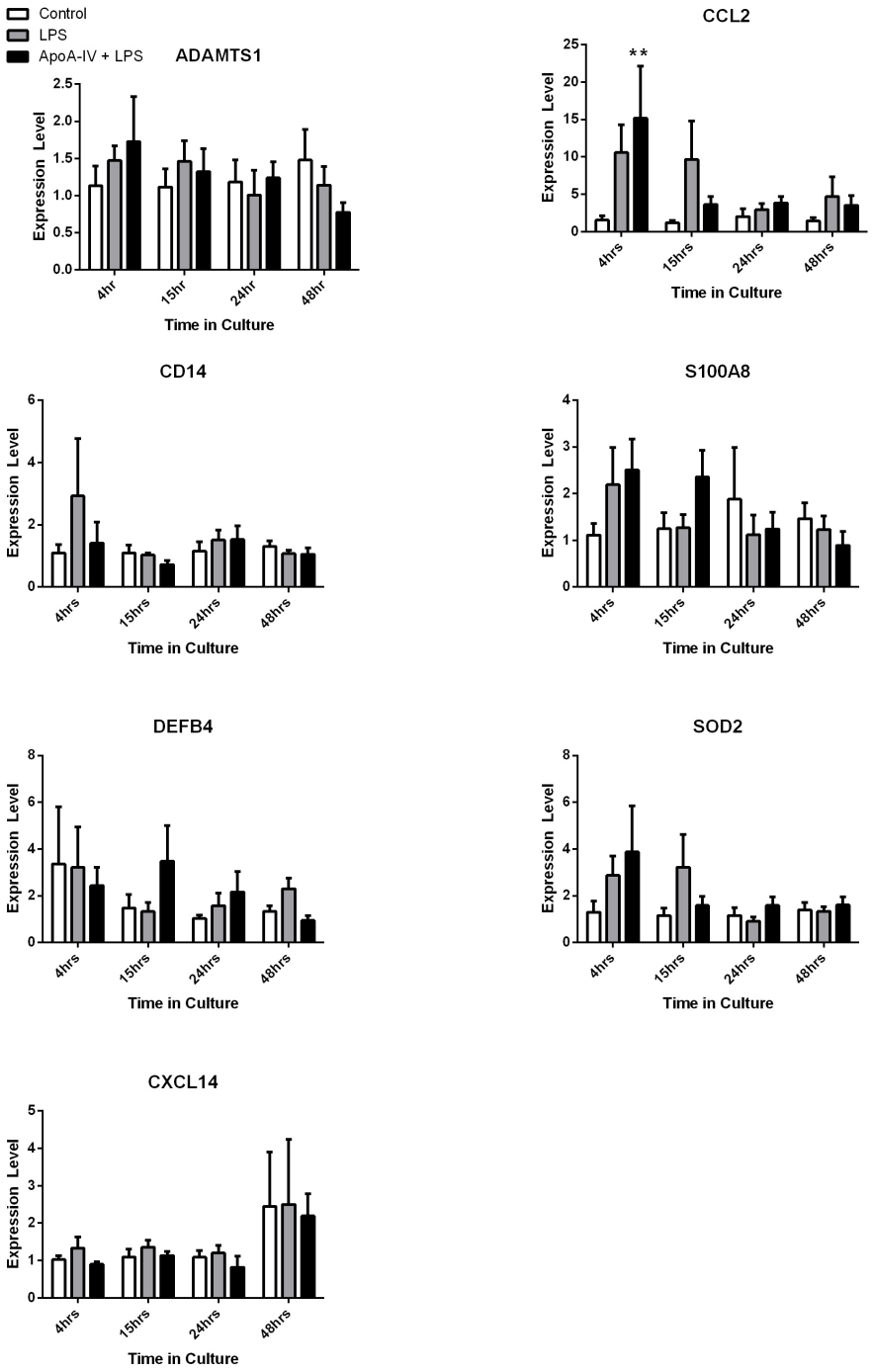


Figure 5.14 – The addition of recombinant human apoA-IV (25µg/ml) with LPS had no significant effect on the expression of the laminitis-associated genes (ADAMTS1, CCL2, CD14, CXCL14, DEFB4, S100A8 and SOD2) when compared with the LPS treatment. Data are presented as means ± SEM.

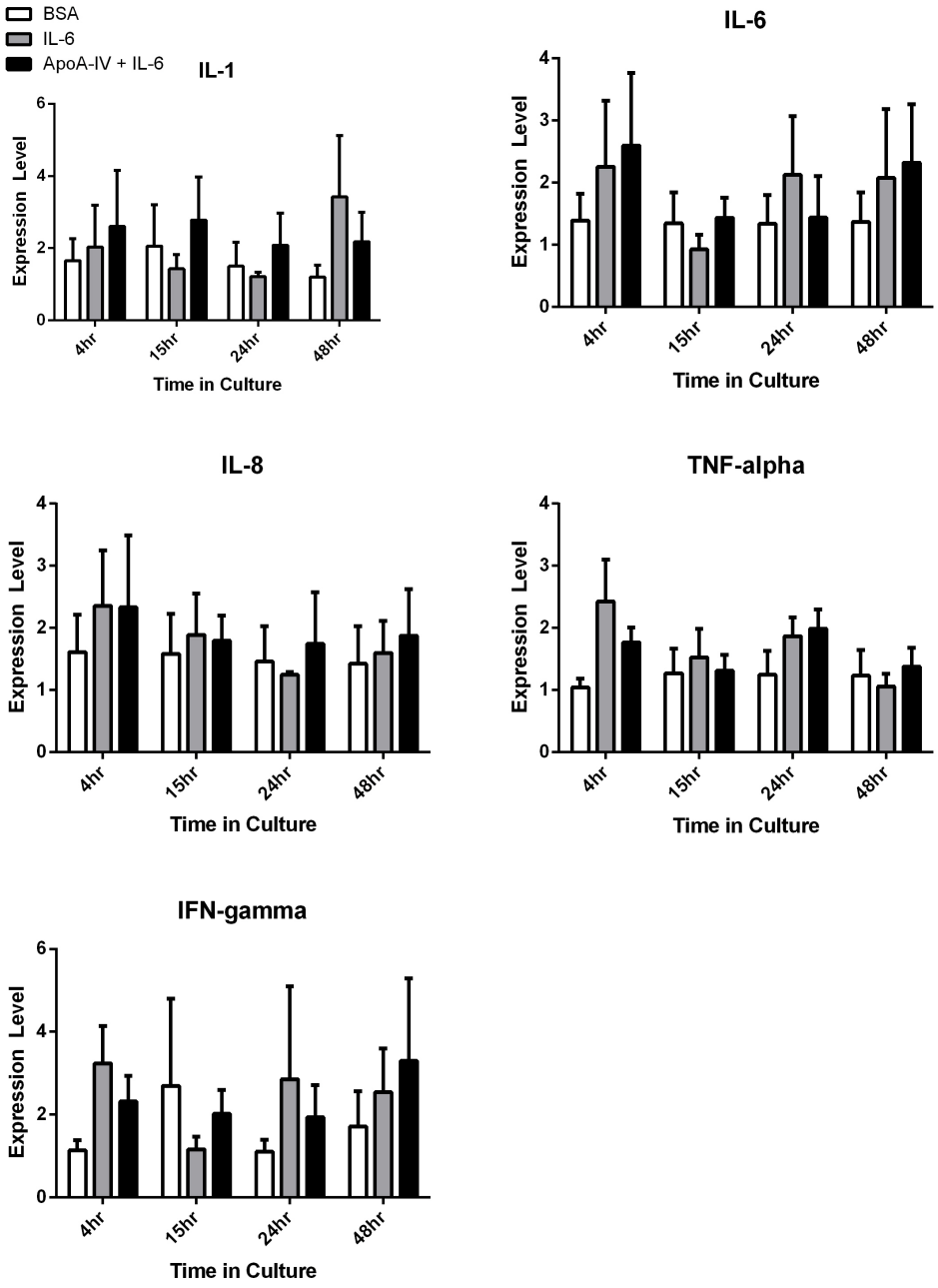


Figure 5.15 – The addition of recombinant human apoA-IV (25µg/ml) with IL-6 had no significant effect on the expression of the inflammatory cytokines (IL-1β, IL-6, IL-8, TNF-α and IFN-γ) when compared to the IL-6 treatment. Data are presented as means ± SEM.

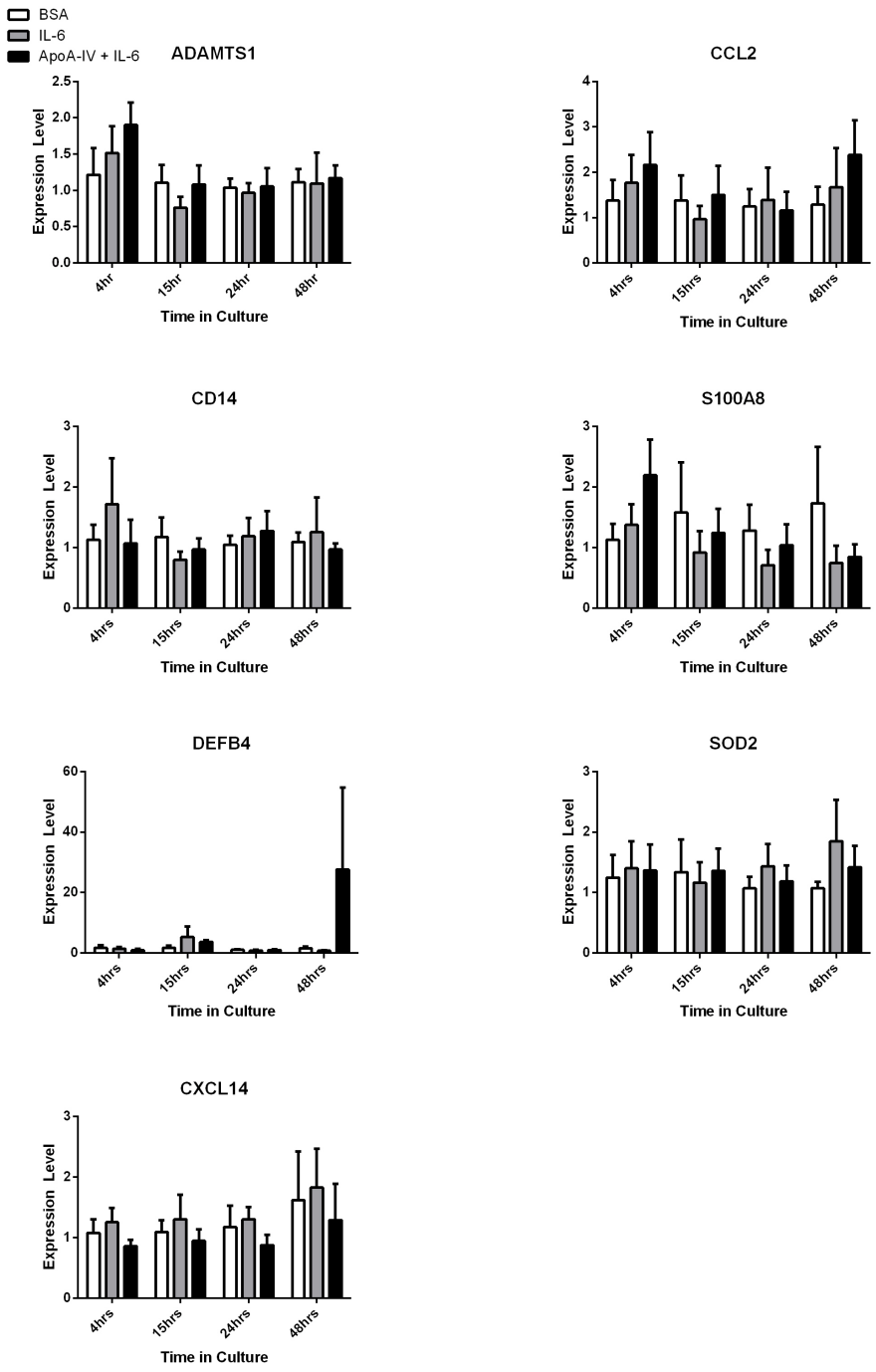


Figure 5.16 – The addition of recombinant human apoA-IV (25µg/ml) with IL-6 had no significant effect on the expression of the laminitis-associated genes (ADAMTS1, CCL2, CD14, CXCL14, DEFB4, S100A8 and SOD2) when compared with the IL-6 treatment. Data are presented as means ± SEM.

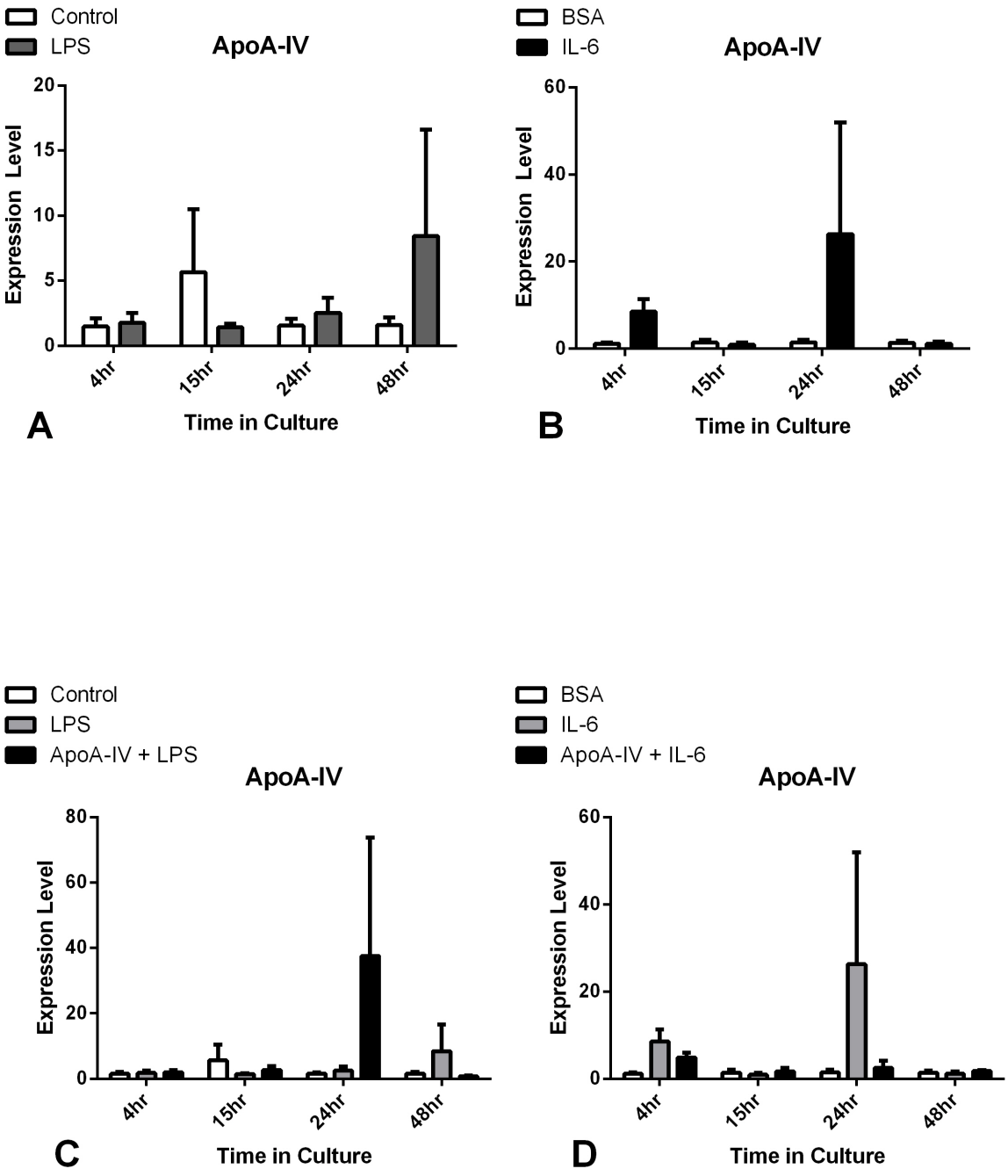
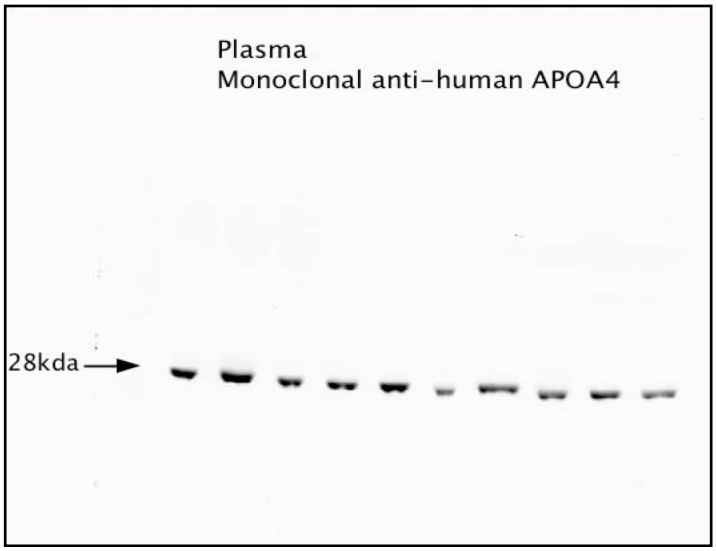


Figure 5.17 – The addition of IL-6 (A) or LPS (B) did not significantly affect the expression of APOA4. The addition of apoA-IV with LPS (C) or IL-6 (D) did not affect the expression of APOA4. Data are presented as means ± SEM.

ApoA-IV isolation*:* A western blot with 1µl plasma in each lane using monoclonal anti-human apoA-IV as a primary antibody and HRP-conjugated goat anti-mouse IgG as secondary antibody identified a protein around 28 kDa (Figure 5.18). The same band was visible when 10µg of laminar tissue proteins were run under the same conditions (Figure 5.18). Previous attempts to identify apoA-IV by mass spec from this band on a 1D gel were unsuccessful (unpublished data). Laminar tissue proteins separated by 2D gel electrophoresis showed different patterns of signal using the monoclonal anti-apoA-IV antibody (Figure 5.19) and a polyclonal anti-apoA-IV antibody (Figure 5.20). Spots with the strongest signal in each gel that were around the previously reported 28 kDa mark and the predicted 43 kDa size of the protein were cut out from a GelCode Blue Safe Protein Stain stained gel run under the same conditions (Figure 5.21). None of these 6 spots contained measurable levels of apoA-IV as determined by mass-spec analysis.

A.



B.

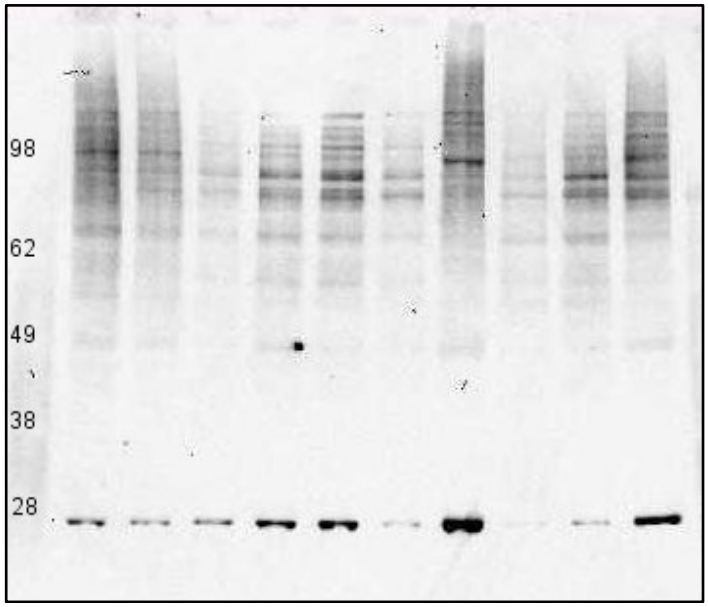


Figure 5.18 – Western blots with monoclonal anti-human apoA-IV primary antibody (Abcam, Cambridge, UK) and HRP-conjugated goat anti-mouse secondary antibody (Abcam, Cambridge, UK). A: equine plasma (1µl/lane) B: equine laminar tissue proteins (10µg/lane).

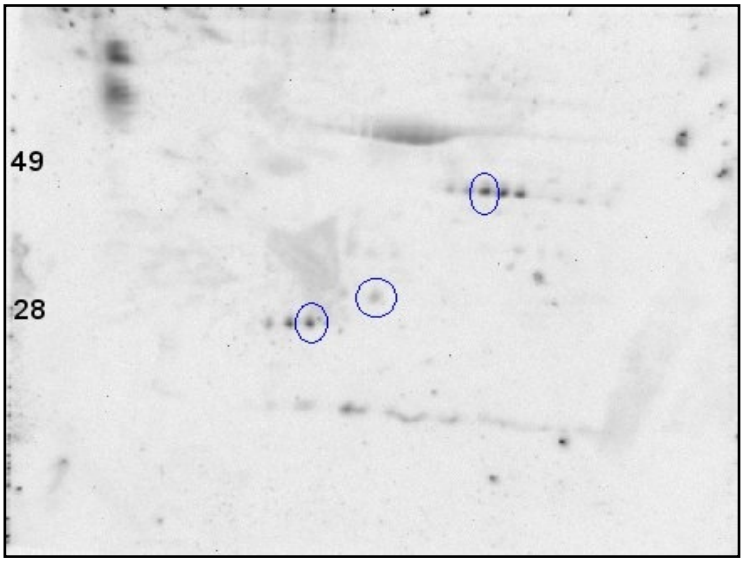


Figure 5.19 – Western blot from a 2D gel loaded with 70 µg of laminar tissue protein with monoclonal anti-human apoA-IV primary antibody (Abcam, Cambridge, UK) and HRP-conjugated goat anti-mouse secondary antibody (Abcam, Cambridge, UK). Blue circles indicate spots that were selected for mass spec analysis.

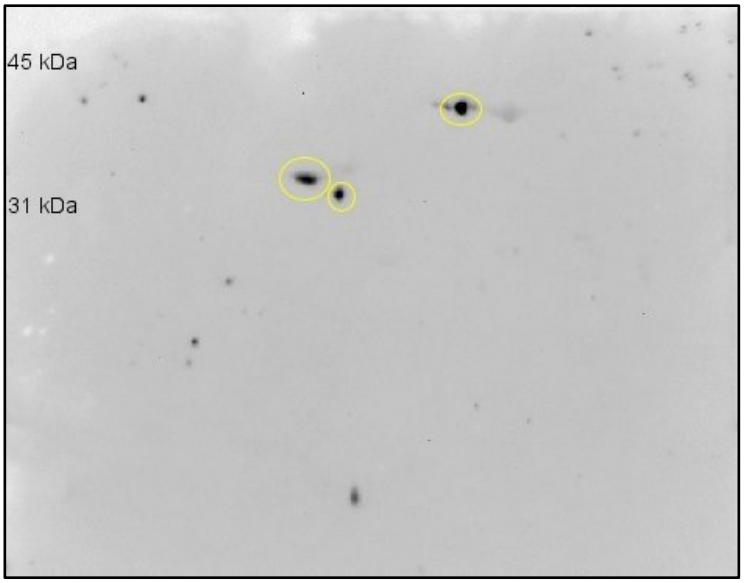


Figure 5.20 – Western blot from a 2D gel loaded with 70 µg of laminar tissue protein with polyclonal anti-human apoA-IV primary antibody (Abcam, Cambridge, UK) and HRP-conjugated goat anti-rabbit secondary antibody (Abcam, Cambridge, UK). Yellow circles indicate spots that were selected for mass spec analysis.

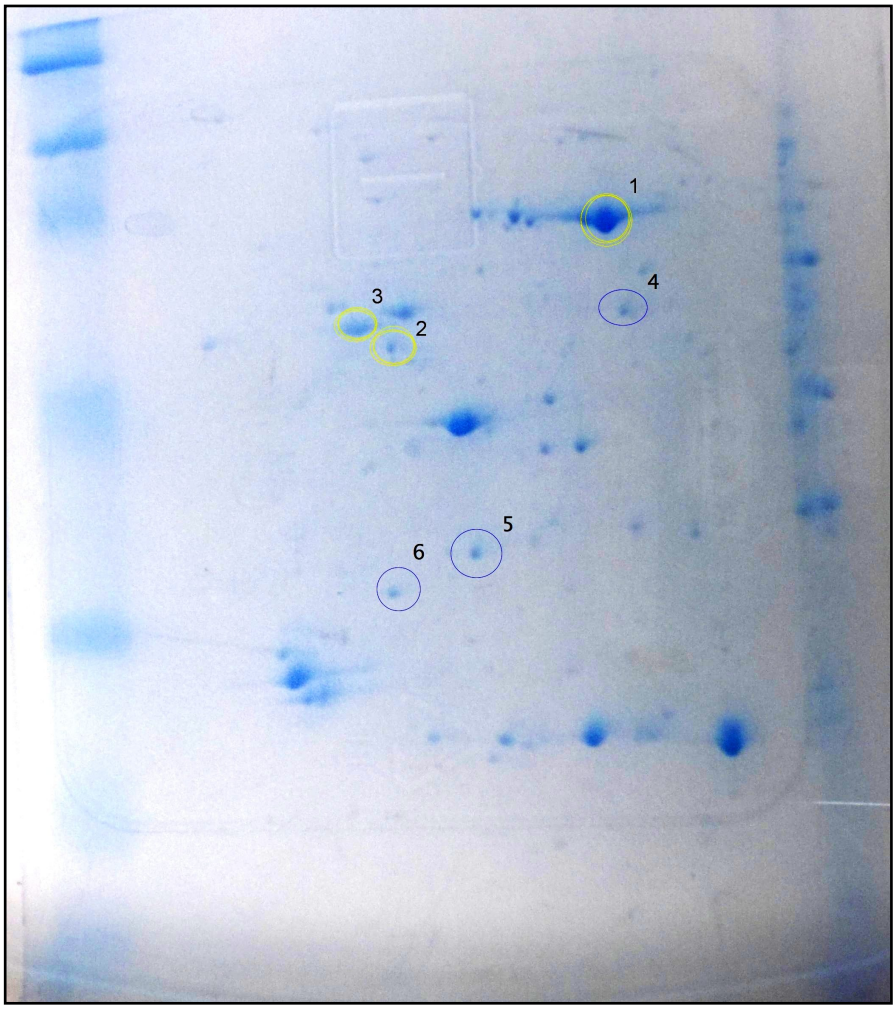
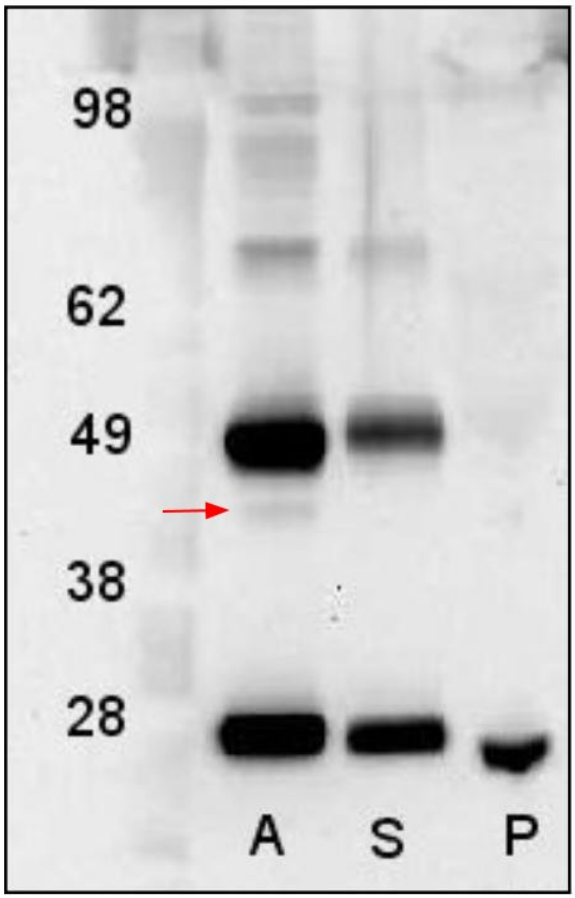


Figure 5.21 – A 2D gel loaded with 70 µg of laminar tissue protein stained with GelCode Blue Safe Protein Stain (Thermo Fisher Scientific Inc., Rockford, IL). Spots from polyclonal antibody western blot are marked in yellow and spots from monoclonal antibody western blot are marked in blue.

Product from an immunoprecipitation using the monoclonal anti-human apoA-IV antibody in equine plasma run on a 4-12% polyacrylamide gel showed one band which was not present in the mouse serum control in both reducing and non-reducing conditions (Figure 5.22). However, it was shown that this band also appeared when the anti-apoA-IV antibody used in the immunoprecipitation was run on a gel as a control, showing that it was most likely a relic of that specific antibody and not its bound product (Figure 5.23). The immunoprecipitation western blots (Figure 5.22) also show a shift of the band in the plasma sample from around 28 kDa in the reducing gel to above the 98 kDa marker in the non-reducing gel. Given that these sizes roughly correspond to the size of IgG light chain alone and an intact IgG antibody, a western blot was conducted on equine plasma 1µl/lane with only the goat anti-mouse IgG secondary antibody to test for cross-reaction. This showed that the band seen in our initial western blot was due to cross-reaction with the secondary antibody (Figure 5.24). A western blot was run with 5µg of affinity purified equine IgG and only the HRP-conjugated goat anti-mouse secondary antibody showed some cross-reaction with the secondary antibody (Figure 5.25).

A.



B.

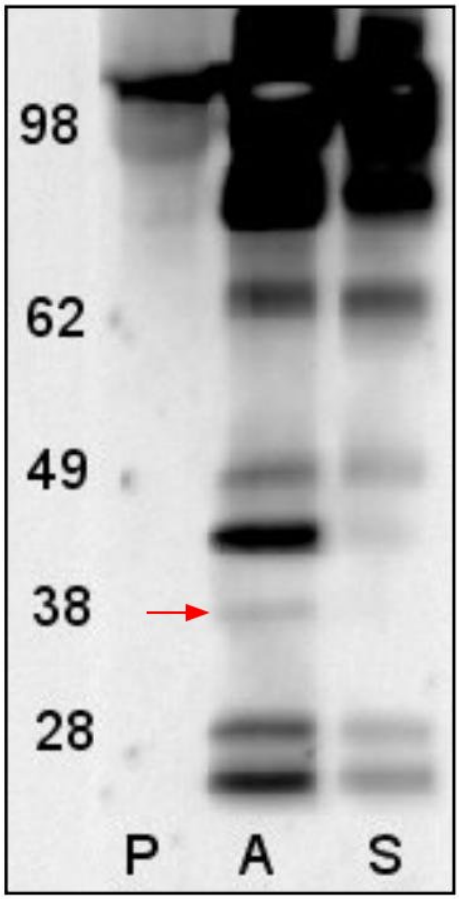


Figure 5.22 – Western blots of IP product run under reducing conditions (A) and non-reducing conditions (B) using monoclonal anti-human apoA-IV primary antibody (Abcam, Cambridge, UK) and HRP-conjugated goat anti-mouse IgG secondary antibody (Abcam, Cambridge, UK). Each IP product lane contains 100µl of precipitated and lyophilized immunoprecipitation elution re-dissolved in loading buffer. Lanes contain A: immunoprecipitation with anti-human apoA-IV monoclonal antibody, B: immunoprecipitation with mouse serum control, and P: equine plasma (1µl/lane). Arrows indicate band present only in immunoprecipitation with antibody and not present in the serum control.

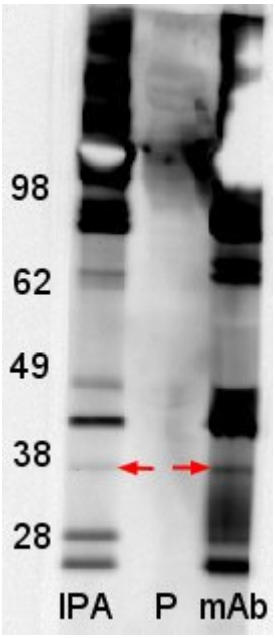


Figure 5.23 – Western blot with immunoprecipitation product from monoclonal anti-human apoA-IV antibody (IPA), 1µl plasma (P), and 5ul of monoclonal anti-human ApoA-IV antibody (mAb). Blot was run using monoclonal anti-human apoA-IV primary antibody (Abcam, Cambridge, UK) and HRP-conjugated goat anti-mouse IgG secondary antibody (Abcam, Cambridge, UK). Arrows indicate band that was present only in immunoprecipitation with antibody and not serum control.

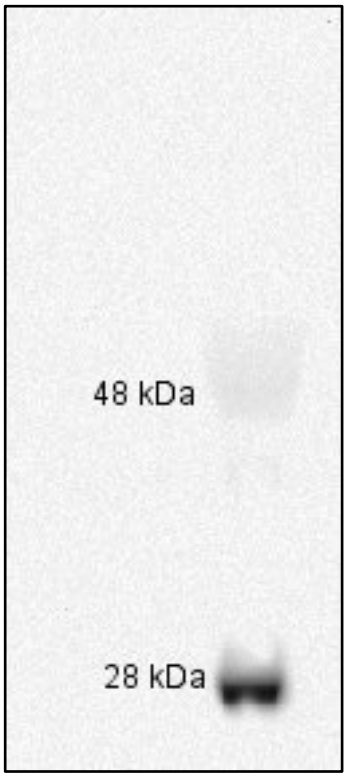


Figure 5.24 – Western blot of equine plasma (1µl/lane) with only goat anti-mouse secondary antibody (Abcam, Cambridge, UK) and no primary antibody.

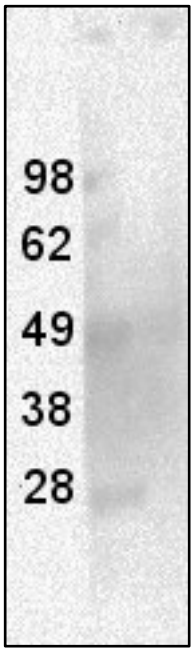


Figure 5.25 – Western blot of affinity purified equine IgG (5µg) using monoclonal anti-human apoA-IV primary antibody (Abcam, Cambridge, UK) and HRP-conjugated goat anti-mouse IgG secondary antibody (Abcam, Cambridge, UK).

Several other anti-IgG antibodies were tested, but all were found to cross-react (Figure 5.26). Running a western blot with only the secondary antibody HRP-conjugated goat anti-rabbit (Abcam) resulted in a number of cross-reacting bands. When a western blot was run on the immunoprecipitation product from monoclonal anti-human apoA-IV (Abcam) using the same primary antibody as that from the immunoprecipitation and HRP-conjugated anti-mouse IgG VeriBlot antibody (Abcam), which is designed to bind only to intact antibody, as a secondary antibody, cross-reaction still occurred under reducing conditions. HRP-conjugated monoclonal anti-human apoA-IV had high background because the HRP-conjugation was conducted in an antibody in ascites fluid, but no bands were seen around the predicted size for apoA-IV. Because these results provide no evidence that apoA-IV is found

at 28 kDa and not the expected 43 kDa on a western blot, no further attempts were made to isolate and sequence the protein.

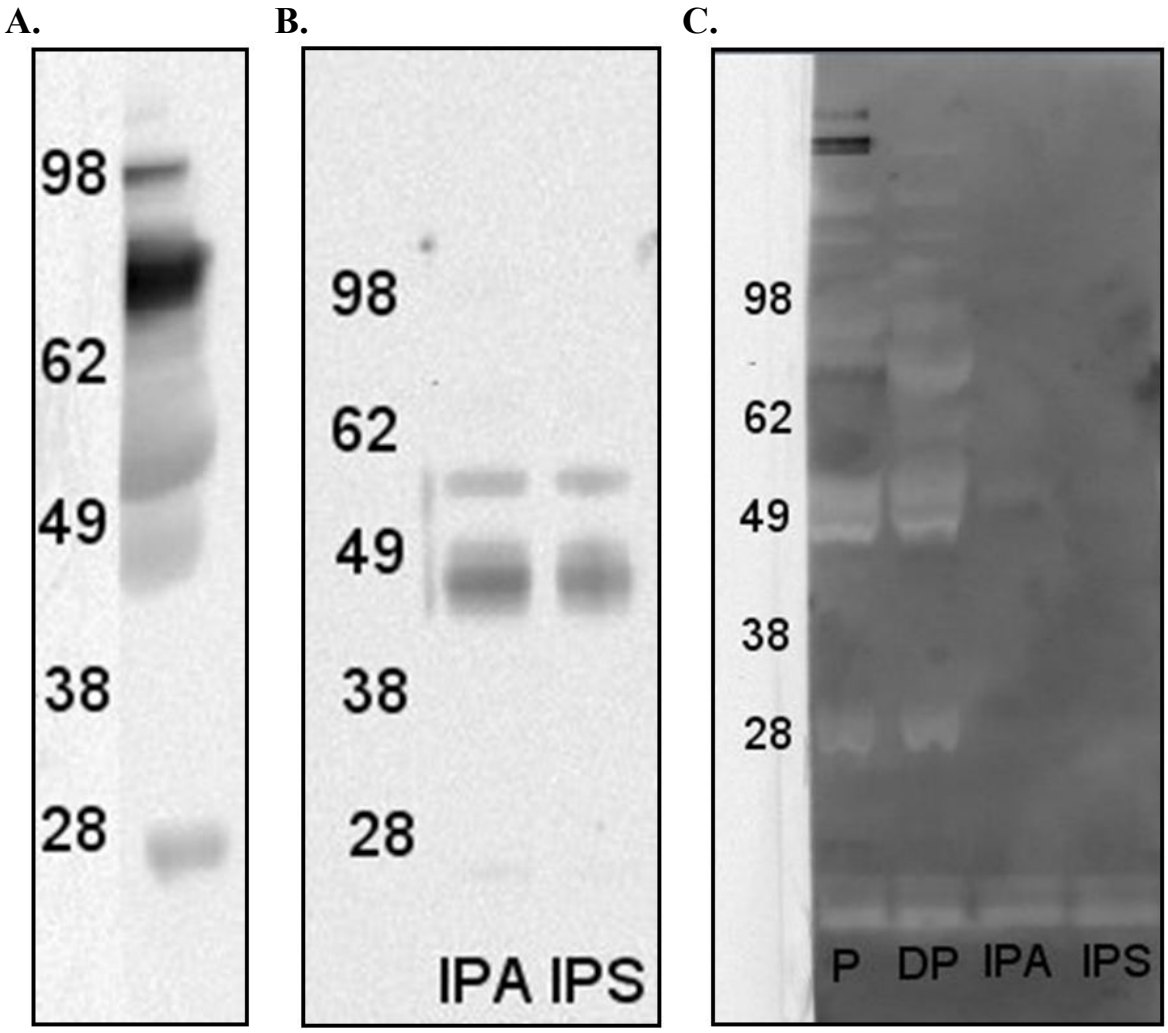


Figure 5.26 – Western blots for apoA-IV using alternative secondary antibodies. A: western blot with 1µl plasma using no primary antibody and HRP-conjugated polyclonal goat-anti rabbit IgG (Abcam, Cambridge, UK) as a secondary antibody. B: western blot with immunoprecipitation product from monoclonal anti-human apoA-IV antibody (IPA) and mouse serum control (IPS) using monoclonal anti-human apoA-IV antibody as a primary antibody and HRP-conjugated anti-mouse IgG VeriBlot antibody (Abcam, Cambridge, UK) as a secondary antibody. C: western blot with 1µl plasma (P), 1µl depleted plasma (DP), immunoprecipitation product from monoclonal anti-human apoA-IV antibody (IPA) and mouse serum control (IPS) using HRP-conjugated monoclonal anti-human apoA-IV as the single antibody.

6. CONCLUSION

6.1 Background

Laminitis is a common equine disease in which inflammation occurs in the laminar tissue, which connects the hoof wall to the distal phalanx. Laminitis has broad impacts in the equine industry, having been estimated by one study to affect 15% of horses at some point in their lives [4], and to cost the industry around 13 million dollars annually [3] in medical expenses and the loss of animals that require euthanasia. The mechanisms behind the inflammation and damage to the laminar tissue during laminitis are not well understood, but two main underlying mechanisms have been suggested in the initiation of the disease: ishchaemia followed by reperfusion into the hoof tissue [2, 44, 76], and the action of an inflammatory agent initiating an inflammatory response in the animal [2, 76]. There has been debate in the literature as to which of these is responsible for causing the laminar tissue degradation seen in laminitis, but both processes are clearly important in laminitis [2, 76], regardless of which is ultimately responsible for initiating the disease process.

Acute laminitis is accompanied by a characteristic bounding digital pulse at the fetlock [2], indicative of vasoconstriction of vessels within the hoof, and reperfusion injuries have been seen in horses affected with the disease [44]. Increases in the production of inflammatory cytokines have been measured in a number of in-vivo acute laminitis studies across the carbohydrate overload [37, 51], black-walnut [49, 50, 52], hyperinsulinemia [53], and oligofructose-overload [38] models of laminitis within the first two days after treatment. These relatively early measurements of increases in inflammatory cytokines indicate that laminar tissue inflammation occurs in the initial stages of the disease. IL-6 has been observed

to be increased in the laminar tissue as early as 12 hours post-induction in the oligofructose overload model of the disease [38]. Although these changes in inflammatory signaling have been observed in a number of studies, the mechanisms underlying this shift to an inflammatory state remain unclear. Suggestions for inflammatory mediators include D-lactate [77] and LPS [24, 25] produced in the gut due to a shift in microbial populations, and the early involvement of the inflammatory cytokine interleukin 6 (IL-6) in the development of the disease [38]. Identifying the causative agent(s) in the development of laminar tissue inflammation is an important step in the understanding of the disease and the eventual development of effective treatment strategies, which are currently extremely limited.

6.2 Tissue culture model

The vast majority of laminitis research today relies on the induction of the disease in live animals. In addition to the large number of animals that must be sacrificed for this type of research, sample groups are typically small and variable in age and health. Here we present a tissue culture model for the disease in which laminar tissue from horses euthanized for reasons unrelated to hoof lameness can be treated with an inflammatory agent to mimic the inflammation found in the laminar tissue during laminitis. This model allows for treated samples to be compared back to untreated control samples from the same animal at each time point, thus reducing the amount of variation introduced due to a heterogeneous sample population. In our model we use Leibovitz's medium because it has been shown previously to maintain tissue morphology in culture [78, 79], and is a minimal medium that better reflects the non-proliferative in vivo state of the laminar tissue. Because this model allows for the direct addition of potential causative agents to the laminar tissue, it allows for the

determination their effects on this particular tissue, eliminating down-stream effects from the agent's interaction with other organ systems. While in-vivo studies are important in providing a picture of the course of the disease over time and a system-wide view of the animal, isolating the laminar tissue is an important step in determining the mechanisms that drive inflammation in this specific tissue.

We were successful in keeping laminar tissue viable throughout the slices over the course of the experiment as determined by MTT staining. Nearly all samples remained free of contamination over the course of the experiment, with only two samples being discarded. These results show that our setup is a viable option for laminar tissue culture, and that experiments can be carried out over multiple days in this model. This would allow our model to be used in studies in which several elements were introduced over time; for example, inflammation could be initiated in the tissue prior to adding a potential anti-inflammatory intervention to test possible drugs and treatments in vitro before moving to live animal models. Inflammatory cytokine expression was increased in the controls over the course of the experiment, likely due to mechanical damage to the tissue during collection. However, as the highest effect was seen at 4 h, it is unlikely that there was a significant difference in inflammatory cytokine protein production after this short a period of time that would affect the tissue culture. Additionally, as we compared treatments to controls for each time point, this culture-effect was accounted for in our analysis.

In the future, this model could also be of use in testing the effects of agents suspected of having a causal role in laminitis on laminar tissue in vitro. Because the laminar tissue is isolated, this model distinguishes between agents that affect the laminar tissue directly to induce inflammation and those that act in other organs of the horse to produce upstream

effects that eventually result in laminar tissue inflammation. This distinction is important because it could help to determine the sequence of events in the development of the disease and provide information as to the level at which potentially causative agents act in the horse.

One current limitation of the model is that it does not simulate the load bearing stress normally exerted on the laminar tissue in a live animal. Given the constant force laminar tissue in live animals is under and the role of load-bearing stress in the development of laminitis [14, 70, 80], future development of the model could include culturing the tissue under conditions of shear force to mimic the mechanical stress exerted on the tissue in vivo. Additionally, it could be useful to further characterize the model histologically in order to determine whether cellular organization remains intact over the course of the experiment in the control treatment and if the histological changes induced in live animals with laminitis can be seen in the treated tissue in our model.

6.3 Effects of LPS and IL-6 on laminar tissue inflammation

We tested two different inflammatory agents, lipopolysaccharide (LPS) and interleukin 6 (IL-6), to compare their effects on gene expression on the laminar tissue in our model to changes in gene expression measured in animals experiencing laminitis. These agents were chosen based on their previous associations with laminitis, and gene expression was measured in genes that had been shown to change consistently across models of laminitis. We found that the addition of LPS was more successful in inducing gene expression patterns mimicking those found in vivo in animals experiencing laminitis than was the addition of IL-6.

LPS is an important structural component in the cell wall of gram-negative bacteria

used by the immune system to identify a bacterial infection. LPS is recognized by toll-like receptor 4 (TLR4), which initiates a signaling pathway leading to the activation of inflammation-related genes [81-83]. In order for the immune system to detect an intrusion of gram-negative bacteria, LPS is initially disassociated from the outer membrane of these bacteria by LPS binding protein (LBP) [84]. Aggregates of dissociated LPS are then bound to CD14, a membrane-bound co-receptor which splits these aggregates into LPS monomers that can more easily bind to toll-like receptor 4 [84]. TLR4 acts in conjunction with a protein called MD-2 to form a complex that binds more readily to LPS than TLR4 alone [84-86]. Once bound to LPS, the TLR4/MD-2 complex aggregates with other LPS-bound complexes to initiate a signaling cascade resulting in the activation of transcription factors including NF_KB [84, 86]. Once NF_KB has been activated, it is translocated into the nucleus where it functions to upregulate the production of inflammatory cytokines and other inflammation-associated genes [84, 86]. LPS is also known to induce polyclonal activation of B-cells through TLR4 [83, 87], but the expression of B-cell specific transcripts have been shown to decrease in horses with induced laminitis [88] and plasma levels of immunoglobulin were unaltered in horses with chronic laminitis [72]. In our study LPS was added alone without the addition of LBP (normally found in the plasma). Although this may have affected the strength of the signaling reaction to LPS, measurable responses in gene expression were still obtained. It was not possible to add equine plasma to our culture as this results in the proliferation of cells in the laminar tissue, an activity that does not occur in vivo and could alter the structure of the laminar tissue.

There is reason to believe that LPS may be involved at some level in the development of laminitis. It has been suggested that a shift in gut bacteria in carbohydrate overload and

pasture associated laminitis leads to an increase in LPS in the gut which is responsible for inducing the inflammation in the laminar tissue seen in laminitis [24]. LPS has been shown to be increased in oligofructose-induced laminitis [24] and in cecal fluid following carbohydrate overload [89]. Additionally, anti-LPS immunoglobulin has been measured to increase following colic [25], a condition that often precedes laminitis. Anti-LPS has also been measured in the plasma of humans with rheumatoid arthritis [90, 91] and been associated with lower mortality following traumatic injury [92] and reducing endotoxemia [93]. However, despite the correlation between increased LPS levels and gastrointestinal disorders and laminitis, intravenous administration of LPS alone is not sufficient to induce laminitis in live animals [76, 94]. Additionally, while there are increases in specific gram-negative species in the gut during laminitis, the overall shift in bacteria has been shown to be towards an increase in gram-positive species [19-21]. This indicates that, while LPS may play a role in laminar tissue inflammation in horses with laminitis, it is not the only factor involved in the initiation and development of the disease. Despite previous research indicating that the production of LPS may be involved in the development of laminitis, its role (if any) in laminitis is not understood, and the effects of LPS on laminar tissue are unknown.

In this study, the addition of LPS to cultured laminar tissue caused the expression of inflammatory cytokines to change after 4 h to mimic the pattern seen in horses experiencing acute laminitis. As in most animal studies, IL-1β, IL-6, and IL-8 were increased at this time point while TNF-α and IFN-γ remained unchanged. In live animal studies IL-1β [49-52], IL-6 [38, 49-51], and IL-8 [49, 50] were also measured to increase in expression across multiple models. As seen in our tissue culture experiment, the expression of TNF-α [50, 51] has been shown to remain unchanged in the laminar tissue during induced laminitis. IFN-γ has also

been shown to remain unchanged in the black walnut [49] and carbohydrate overload [51] models of laminitis, although it was shown to increase in the oligofructose overload model of the disease [49]. The high correlation between the expression levels of cytokines measured to be significantly affected by LPS (IL-1β, IL-6, and IL-8) provides evidence that our measurement were robust and indicative of an underlying biological response. Additionally, correlations involving the cytokines that were not significantly affected (TNF-α and IFN-γ) were much lower, further indicating that the expression of these genes was probably not responding in a clear pattern to the addition of LPS.

The observed increases in IL-1β, IL-6, and IL-8 can be explained by the action of the transcription factor NF_KB (Figure 6.1), which is relocated into the nucleus following TLR4 activation by LPS. NF_KB is known to have a functional binding site for IL-1β [95] and to regulate the expression of IL-6 [96, 97] and IL-8 [98, 99]. However, NF_KB has also been implicated in the transcriptional regulation of TNF-α [100, 101] and IFN-γ [102, 103], which were not significantly changed at any time point in this experiment. Further research on the factors regulating inflammatory cytokine production during laminitis may shed light on why the pro-inflammatory cytokines TNF-α and IFN-γ were not upregulated in the disease or in our model.

Increases in IL-1β, IL-6, and IL-8 expression in the laminar tissue during laminitis could play an important role in the recruitment of leukocytes into the tissue. IL-1β is known to act in recruiting neutrophils [104, 105], which have been shown to be increased in the laminar tissues in horses with laminitis [7, 38, 72, 106]. This increase in neutrophils is associated with increased levels of metalloproteinases [29, 30], which may contribute to tissue damage in the laminar tissue during laminitis. In addition, IL-1β is involved in

collagen production [104], of note because collagen is one of the major components of the basement membrane that is degraded during laminitis, and blocking the production of IFN-γ [104, 107], which could explain the lack of response in this particular cytokine during laminitis. IL-6 plays an important role in initiating an immune response. It has been shown to be involved in the recruitment of leukocytes [108] and has been suggested to play such a role in laminitis [38]. Additionally, IL-8 is well known to play a role in the recruitment of neutrophils [109, 110]. Taken together, increased expression of these cytokines indicates an inflammatory response in the laminar tissue, recruiting leukocytes (especially neutrophils) into the tissue and ramping up the local immune response.

In addition to the increased expression of several inflammatory cytokines, two of the seven laminitis-associated genes we measured (CCL2 and SOD2) were increased over the course of the experiment, although differences were not significant at any specific time point. CCL2 and superoxide dismutase (SOD) were increased in the laminar tissue of horses with black walnut extract induced laminitis [88], showing CCL2 and a superoxide dismutase enzyme to be affected in multiple live-animal studies. CCL2 is known to recruit monocytes and macrophages and to be released in response to oxidative stress [111]. SOD2 functions to transform superoxide (which is toxic to cells) into hydrogen peroxide and diatomic oxygen and is known to be produced by neutrophils [112, 113]. The increased expression of SOD2 in the laminar tissue in horses with laminitis [71] could be a response to mitigate the damaging effects of oxidative stress introduced by neutrophils on the laminar tissue.

The expression pattern of the laminitis-associated genes can also be explained through the activation of NF_KB (Figure 6.1). CCL2 [114-116] and SOD2 [117, 118] are also both target genes of NF_KB while the other, unaffected, laminitis-associated genes have not been

identified in the literature as targets for this transcription factor. Although our results show that LPS exposure is not a perfect model for laminitis, the cytokine expression profile resulting from LPS treatment may produce a viable model for certain future studies, particularly those that are expected to act through the NF_KB pathway.

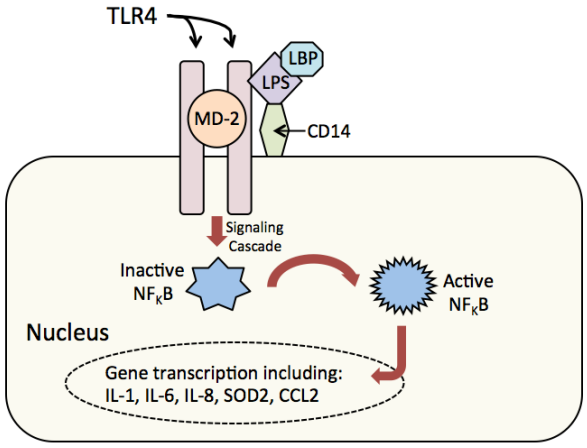


Figure 6.1 – Gene expression regulation through TLR4. LPS signaling through the TLR4 receptor activates NF_KB which is translocated to the nucleus where it can upregulate the expression of the inflammatory cytokines and laminitis associated genes measured to increase in this study in addition to many other inflammation-related genes.

Application of the second inflammatory agent used in this study, IL-6, did not produce changes in gene expression in the laminar tissue culture consistent with those found in the laminar tissue of horses experiencing laminitis. IL-6 is an inflammatory cytokine involved in a wide number of biological processes including inflammation, oncogenesis, hematopoiesis, and immune regulation [119]. The protein functions by binding to the IL-6 receptor (IL-6R) which leads to the homodimerization of gp130, a cell surface protein that serves as a signal transducer for a number of cytokines including IL-6 [119-122]. The binding of IL-6 to the IL-6R and subsequent homodimerization of gp130 leads to the activation of JAKs which then

activates STAT1 and STAT3. After activation these proteins are able to form homodimers or heterodimers that, when transported to the nucleus, induce transcription of target genes [119, 122-125].

IL-6 production has been implicated in the development of laminitis [38] and has was increased in the black walnut [49, 50], oligofructose [38], and carbohydrate overload [51] models of the disease. IL-6 was shown to induce changes in matrix metalloproteinase expression in vitro in a previous study [38]. In this case recombinant equine IL-6 was added to laminar tissue explants 5mm x 5mm at varying concentrations. At a concentration of 100ng/ml and 500ng/ml, IL-6 was seen to increase the expression of pro MMP-9 although the changes were lost at a concentration of 1000ng/ml, and no changes were observed in the concentration of pro MMP-2 at any concentration of IL-6. The authors suggest that IL-6 may act in the recruitment of leukocytes into the laminar tissue, and that it may do so through an increase in the production of MMP-9. However, the role of IL-6 in laminar tissue inflammation is currently not understood, and in this study we examined its effects on laminar tissue in isolation to determine if the protein alone had the ability to induce inflammation in cultured laminar tissue.

We found that the addition of IL-6 by itself was not sufficient to significantly affect the expression of any of the measured inflammatory cytokines (IL-1β, IL-6, IL-8, TNF-α, or IFN-γ). IL-6 also did not affect the expression of the laminitis-associated genes ADAMTS1, CCL2, CD14, CXCL14, DEFB4, S100A8, or SOD2. Our results suggest that IL-6 alone is not sufficient in driving laminar tissue inflammation. However, this protein could still be important in the development of laminitis through the recruitment of leukocytes, as previously postulated [38]. Our results support the idea that the role of IL-6 in laminitis is

related to the involvement of factors not normally found in the laminar tissue as If IL-6-mediated recruitment of leukocytes or other factors into the laminar tissue would not be reflected in our model. Additionally, the large inter-animal variation in our study may have contributed to these results due to the high standard error in our measurements and future studies with larger numbers of animals may yield less variable results.

This variation could also be indicative of physiological differences between animals in susceptibility to developing laminar tissue inflammation. In several previous in-vivo laminitis studies, a minority of horses were considered "non-responders" as they never developed the disease despite undergoing the same treatment as affected animals [6, 20, 36, 51, 126]. In our experiment, we observed variation in the magnitude of the response to the addition of LPS and IL-6. At 4 h, when the maximal effect was measured, horse number five expressed notably lower levels of inflammatory cytokines in response to both LPS and IL-6 than the other animals. Additionally, this animal showed lower levels of correlation between the expression of different inflammatory cytokines. This raises the possibility that some horses in our model may not respond to inflammatory agents in their laminar tissue to the same degree as the majority, possibly mimicking the "non-responders" found in in vivo trials. Although further studies would be needed to confirm the presence of non-responders in our tissue model, given the relatively small number of animals in our trial and variation in age, breed, and sex, these animals could prove useful in understanding how inflammation is initiated in the laminar tissue and why some animals are naturally more susceptible or resistant to the development of laminitis than others.

6.4 Role of apoA-IV in laminar tissue inflammation

Apolipoproteins are proteins associated with lipoproteins, particles responsible for the transport of lipids through the body. Apolipoprotein A-IV (apoA-IV) is associated with high-density lipoprotein, and, although its primary function is not established, it has been shown to be involved in a number of diverse processes including appetite regulation [63], anti-oxidant activity [127], amyloid B clearing [65], and anti-inflammatory activity [57]. In a previous study [56], apoA-IV was shown to be increased by over two-fold in the plasma of horses with chronic laminitis when compared to healthy controls. The protein has also been associated with two chronic inflammatory diseases in humans, ankylosing spondylitis [68], and rheumatoid arthritis [67]. Additionally, it has been shown to have an anti-inflammatory role in mitigating experimentally induced colitis in mice [57].

Currently, the role of apoA-IV in laminitis is not known, but its association with chronic laminitis and human inflammatory diseases make apoA-IV an important protein to investigate further in the pathogenesis of laminitis. Thus, we conducted an in-vitro experiment to test the effect of apoA-IV on the expression of inflammatory cytokines and laminitis associated genes in laminar tissue alone and in the presence of two inflammatory agents. We also measured the response of APOA4 expression to the addition of the inflammatory mediators LPS and IL-6. Because very little is currently known about the production, distribution, and function of apoA-IV in the horse, we conducted a tissue panel and quantified expression levels of APOA4 at the RNA level to determine its distribution in the horse.

To test the effects of apoA-IV on the laminar tissue under normal and inflamed conditions we added recombinant human apoA-IV to tissue with and without LPS and IL-6.

We found no effect of adding apoA-IV to laminar tissue under control conditions or in addition to either LPS or IL-6. There was no significant effect of apoA-IV treatment in any case on the expression of inflammatory cytokine genes or laminitis associated genes in the cultured laminar tissue. This indicates that apoA-IV is not responsible for directly modulating laminar tissue inflammation under normal or inflamed conditions. However, it remains possible that apoA-IV is an important mediator of laminitis, but acts through a mechanism not directly linked to the laminar tissue itself. Given the association between metabolic syndrome and laminitis, it is interesting to note that apoA-IV has been suggested to play a role in reducing oxidative stress during metabolic syndrome [128]. It is also known to reduce the hepatic lipid burden during hepatic steatosis [129], a problem associated with metabolic syndrome, by promoting lipoprotein particle expansion. ApoA-IV is increased in the plasma following 24 hours of fasting in mice [130], raising the possibility that it may be increased in response to a calorie-restricted diet given to horses with metabolic syndrome. The function of apoA-IV in chronic inflammatory disease remains to be elucidated, and further studies in this area are merited to determine whether this protein has any effect on the inflammatory state in these individuals.

The addition of either LPS or IL-6 to tissue culture had no effect on the expression of APOA4 at any time point. However, the expression levels were very low in the laminar tissue across all treatments and time points. This low expression may have contributed to the variability in expression between horses and high standard error. Given the low level of expression in the laminar tissue it is unlikely that apoA-IV is synthesized in substantial quantities in this tissue. More likely, the increase in apoA-IV seen in horses with chronic laminitis is due to an increase in apoA-IV production in the liver and/or small intestine that is

secreted into the plasma to account for the increased levels of plasma apoA-IV. If apoA-IV is important in modulating laminar tissue inflammation during laminitis, it is most likely that this protein is transported in the blood to the hoof where it is able to act on laminar tissue.

Our tissue panel results showed apoA-IV to be measurably expressed in all tissues examined except the pancreas. However, the gene is much more highly expressed in the liver than in any other tissue, although notable quantities are also expressed in the small intestine. The high relative expression levels in the liver are of interest because not all animals synthesize apoA-IV in the liver. In humans, synthesis of apoA-IV is restricted to the small intestine [63] and, while the protein is produced in both the liver and small intestine in rats, the synthesis is higher in the small intestine than the liver in these animals [131]. The much higher relative expression levels found in the liver than in the intestine in most of our animals indicates that horses may balance the synthesis of apoA-IV differently between these organs than other animals previously studied. Hepatic apoA-IV expression has been associated with increased lipoprotein particle size [129], response to fasting [130], and suppression of glucose production [132], providing a number of possibilities for its role in the liver in horses. Because of this result, further studies on apoA-IV at the protein level and on its synthesis in the liver and small intestine of the horse are merited.

ApoA-IV expression levels between organs was not uniform between the horses, however, with one animal showing much lower expression in the liver and higher expression in the small intestine than the other animals. In this case expression in the small intestine outweighed expression in the liver, showing that there is potential for great variation in production levels between these organs. It is worth noting that we did not have any information about the feeding schedule of the animals included in this study and that the

production of apoA-IV by the small intestine is known to increase after lipid feeding in rats [63, 133-136] and humans [62, 63]. However, the effects of feeding on apoA-IV synthesis in horses remains unknown. Given that the production of apoA-IV is heavily tied to feeding, it is possible that differences in feeding times between the animals used in this study contributed to the altered expression pattern seen in one individual. Further studies are needed to determine how apoA-IV synthesis is regulated in the horse, particularly given the potentially high contribution of the liver in this animal.

Because of size discrepancies between the reported and predicted apoA-IV sequence in the horse, we attempted to isolate and sequence apoA-IV to detect any modifications from its predicted sequence. ApoA-IV has been previously reported to be around 30 kDa in the horse [56]. This is in contrast to the size of the protein in other animals (46 kDa in humans [59], 46 kDa in rats [58], and 44-45 kDa in dogs [59]) and the size predicted for the horse by ExPASy ProtParam [61] of 43.3 kDa based on the predicted equine apoA-IV protein sequence from the NCBI protein sequence database [60] (reference sequence: XP_001502503.1). To address this discrepancy and search for any post-transcriptional or post-translational modifications we attempted to isolate the protein.

Our results provide no evidence that apoA-IV is smaller than the predicted sequence of 43.3 kDa. Attempts to isolate the protein using 2D electrophoresis or immunoprecipitation with anti-human apoA-IV antibodies were unsuccessful. Additionally it was shown that the antibodies used previously to measure apoA-IV expression were showing cross-reaction with a different protein, potentially equine IgG, and do not detect apoA-IV. Attempts to find a secondary antibody that would not cross-react with equine plasma were unsuccessful. We HRP-conjugated the original anti-human apoA-IV antibody, but were not able to identify

equine apoA-IV in a western blot with equine plasma using this antibody. This indicates that the anti-human apoA-IV antibody is not able to recognize equine apoA-IV due to differences between the human and equine proteins.

ApoA-IV is still likely to be increased during equine laminitis as a previous study [56] identified it in a spot on a 2D DIGE gel shown that was more highly expressed in horses with chronic laminitis than in controls. However, in order to verify this result with ELISA, it would be necessary to produce antibodies specific for equine apoA-IV as commercially available anti-human apoA-IV antibodies were not able to bind the equine protein in our western blots. The lack of equine reagents commercially available was a limiting factor in this study, but future work with equine apoA-IV remains to be done. Further studies should be conducted to confirm the increased concentration of apoA-IV in the plasma of horses with laminitis. These studies would need to involve producing the equine protein in-vitro and raising antibodies against it that could be used to measure the plasma concentration of apoA-IV in horses by ELISA. These initial studies verifying an increase in the plasma levels of apoA-IV in horses with laminitis would need to be completed before further studies examining the role of apoA-IV in laminitis could be justified.

REFERENCES

1. Sloet van Oldruitenborgh-Oosterbaan, M.M., *Laminitis in the horse: a review.* Vet Q, 1999. **21**(4): p. 121-7.

2. Katz, L.M. and S.R. Bailey, *A review of recent advances and current hypotheses on the pathogenesis of acute laminitis.* Equine Vet J, 2012. **44**(6): p. 752-61.

3. Moore, R.M., Bud, and M. Jenne, *"Vision 20/20 – Conquer Laminitis by 2020".* Journal of Equine Veterinary Science, 2010. **30**(2): p. 74-76.

4. USDA, *Lameness and Laminitis in U.S. Horses.* USDA:APHIS:VS, CEAH, National Animal Health Monitoring System, 2000.

5. Hampson, B.A., et al., *The feral horse foot. Part B: radiographic, gross visual and histopathological parameters of foot health in 100 Australian feral horses.* Aust Vet J, 2013. **91**(1-2): p. 23-30.

6. Hurley, D.J., et al., *Dynamic changes in circulating leukocytes during the induction of equine laminitis with black walnut extract.* Vet Immunol Immunopathol, 2006. **110**(3-4): p. 195-206.

7. de la Rebiere de Pouyade, G. and D. Serteyn, *The role of activated neutrophils in the early stage of equine laminitis.* Vet J, 2011. **189**(1): p. 27-33.

8. Hood, D.M., *Laminitis as a systemic disease.* Vet Clin North Am Equine Pract, 1999. **15**(2): p. 481-94, viii.

9. Rita de Cassia de Lima Sampaio, J.C.C., Jose Correa de Lacerda Neto, Julieta Rodini Engracia de Moraes, *Histologic alterations of the basement membrane of the dermal and epidermal layers of the hoof in horses with acute laminitis.* Int. J. Morphol., 2004. **22**(4): p. 307-312.

10. Collins, S.N., et al., *The lamellar wedge.* Vet Clin North Am Equine Pract, 2010. **26**(1): p. 179-95.

11. Pollitt, C.C. and M.B. Visser, *Carbohydrate alimentary overload laminitis.* Vet Clin North Am Equine Pract, 2010. **26**(1): p. 65-78.

12. Geor, R.J., *Pasture-associated laminitis.* Vet Clin North Am Equine Pract, 2009. **25**(1): p. 39-50, v-vi.

13. Hood, D.M., *The pathophysiology of developmental and acute laminitis.* Vet Clin North Am Equine Pract, 1999. **15**(2): p. 321-43.

14. Virgin, J.E., et al., *Incidence of support limb laminitis in horses treated with half limb, full limb or transfixation pin casts: a retrospective study of 113 horses (2000-2009).* Equine Vet J Suppl, 2011(40): p. 7-11.

15. Belknap, J.K. and S.J. Black, *Sepsis-related laminitis.* Equine Vet J, 2012. **44**(6): p. 738-40.

16. Belknap, J.K., J.N. Moore, and E.C. Crouser, *Sepsis-From human organ failure to laminar failure.* Vet Immunol Immunopathol, 2009. **129**(3-4): p. 155-7.

17. Wylie, C.E., et al., *Risk factors for equine laminitis: a systematic review with quality appraisal of published evidence.* Vet J, 2012. **193**(1): p. 58-66.

18. Johnson, P.J., et al., *Laminitis and the equine metabolic syndrome.* Vet Clin North Am Equine Pract, 2010. **26**(2): p. 239-55.

19. Milinovich, G.J., et al., *Changes in equine hindgut bacterial populations during oligofructose-induced laminitis.* Environ Microbiol, 2006. **8**(5): p. 885-98.

20. Milinovich, G.J., et al., *Fluorescence in situ hybridization analysis of hindgut bacteria associated with the development of equine laminitis.* Environ Microbiol, 2007. **9**(8): p. 2090-100.

21. Milinovich, G.J., et al., *Microbial ecology of the equine hindgut during oligofructose-induced laminitis.* ISME J, 2008. **2**(11): p. 1089-100.

22. Milinovich, G.J., et al., *Streptococcus henryi sp. nov. and Streptococcus caballi sp. nov., isolated from the hindgut of horses with oligofructose-induced laminitis.* Int J Syst Evol Microbiol, 2008. **58**(Pt 1): p. 262-6.

23. Mallem, M.Y., et al., *Evaluation of the role of superoxide anions in endotoxin-induced impairment of beta-adrenoceptor-mediated vasodilation in equine digital veins.* Am J Vet Res, 2010. **71**(7): p. 773-9.

24. Bailey, S.R., et al., *Plasma concentrations of endotoxin and platelet activation in the developmental stage of oligofructose-induced laminitis.* Vet Immunol Immunopathol, 2009. **129**(3-4): p. 167-73.

25. Morris, D.D. and J.N. Moore, *Antibody titres to core lipopolysaccharides in horses with gastrointestinal disorders which cause colic.* Equine Vet J Suppl, 1989(7): p. 29-32.

26. Garner, M.R., J.F. Flint, and J.B. Russell, *Allisonella histaminiformans gen. nov., sp. nov. A novel bacterium that produces histamine, utilizes histidine as its sole energy source, and could play a role in bovine and equine laminitis.* Syst Appl Microbiol, 2002. **25**(4): p. 498-506.

27. Chavance, J., *Histamine theory and treatment of laminitis.* Vet Med, 1946. **41**: p. 199-201.

28. Yin, C., et al., *Tissue concentrations of 4-HNE in the black walnut extract model of laminitis: indication of oxidant stress in affected laminae.* Vet Immunol Immunopathol, 2009. **129**(3-4): p. 211-5.

29. Loftus, J.P., J.K. Belknap, and S.J. Black, *Matrix metalloproteinase-9 in laminae of black walnut extract treated horses correlates with neutrophil abundance.* Vet Immunol Immunopathol, 2006. **113**(3-4): p. 267-76.

30. Loftus, J.P., et al., *Leukocyte-derived and endogenous matrix metalloproteinases in the lamellae of horses with naturally acquired and experimentally induced laminitis.* Vet Immunol Immunopathol, 2009. **129**(3-4): p. 221-30.

31. Garner, H.E., et al., *Changes in the caecal flora associated with the onset of laminitis.* Equine Vet J, 1978. **10**(4): p. 249-52.

32. Duncan, S.G., et al., *Alterations in coagulation and hemograms of horses given endotoxins for 24 hours via hepatic portal infusions.* Am J Vet Res, 1985. **46**(6): p. 1287-93.

33. de Laat, M.A., et al., *Equine laminitis: induced by 48 h hyperinsulinaemia in Standardbred horses.* Equine Vet J, 2010. **42**(2): p. 129-35.

34. de Laat, M.A., et al., *Equine laminitis: comparative histopathology 48 hours after experimental induction with insulin or alimentary oligofructose in standardbred horses.* J Comp Pathol, 2011. **145**(4): p. 399-409.

35. Obel, N., *Studies on the histopathology of acute laminitis*. 1948, Uppsala,: Almqvist & Wiksells boktr. 95 p.

36. Riggs, L.M., et al., *Comparison of characteristics and enzymatic products of leukocytes in the skin and laminar tissues of horses administered black walnut heartwood extract or lipopolysaccharide.* Am J Vet Res, 2009. **70**(11): p. 1383-90.

37. Faleiros, R.R., et al., *Laminar leukocyte accumulation in horses with carbohydrate overload-induced laminitis.* J Vet Intern Med, 2011. **25**(1): p. 107-15.

38. Visser, M.B. and C.C. Pollitt, *Lamellar leukocyte infiltration and involvement of IL-6 during oligofructose-induced equine laminitis development.* Vet Immunol Immunopathol, 2011. **144**(1-2): p. 120-8.

39. Pollitt, C.C., *Equine Laminitis - Current Concepts*, A.G.R.I.R.a.D. Corporation, Editor. 2008, Rural Industries Research and Development Corporation: RIRDC Innvocation for rural Australia.

40. Visser, M.B. and C.C. Pollitt, *The timeline of lamellar basement membrane changes during equine laminitis development.* Equine Vet J, 2011. **43**(4): p. 471-7.

41. Pollitt, C.C., *The basement membrane at the equine hoof dermal epidermal junction.* Equine Vet J, 1994. **26**(5): p. 399-407.

42. Pollitt, C.C. and M. Daradka, *Equine laminitis basement membrane pathology: loss of type IV collagen, type VII collagen and laminin immunostaining.* Equine Vet J Suppl, 1998(26): p. 139-44.

43. Pollitt, C.C., *Basement membrane pathology: a feature of acute equine laminitis.* Equine Vet J, 1996. **28**(1): p. 38-46.

44. Hood, D.M., et al., *The role of vascular mechanisms in the development of acute equine laminitis.* J Vet Intern Med, 1993. **7**(4): p. 228-34.

45. Kuwano, A., et al., *Unilateral basement membrane zone alteration of the regenerated laminar region in equine chronic laminitis.* J Vet Med Sci, 2005. **67**(7): p. 685-91.

46. Onishi, J.C., et al., *Chronic laminitis is associated with potential bacterial pathogens in the laminae.* Vet Microbiol, 2012. **158**(3-4): p. 329-36.

47. Carter, R.A., et al., *Decreased expression of p63, a regulator of epidermal stem cells, in the chronic laminitic equine hoof.* Equine Vet J, 2011. **43**(5): p. 543-51.

48. Jones, E., et al., *Neuropathic changes in equine laminitis pain.* Pain, 2007. **132**(3): p. 321-31.

49. Belknap, J.K., et al., *Lamellar pro-inflammatory cytokine expression patterns in laminitis at the developmental stage and at the onset of lameness: innate vs. adaptive immune response.* Equine Vet J, 2007. **39**(1): p. 42-7.

50. Loftus, J.P., et al., *Early laminar events involving endothelial activation in horses with black walnut- induced laminitis.* Am J Vet Res, 2007. **68**(11): p. 1205-11.

51. Leise, B.S., et al., *Laminar inflammatory gene expression in the carbohydrate overload model of equine laminitis.* Equine Vet J, 2011. **43**(1): p. 54-61.

52. Fontaine, G.L., et al., *Expression of interleukin-1beta in the digital laminae of horses in the prodromal stage of experimentally induced laminitis.* Am J Vet Res, 2001. **62**(5): p. 714-20.

53. de Laat, M.A., et al., *Toll-like receptor and pro-inflammatory cytokine expression during prolonged hyperinsulinaemia in horses: Implications for laminitis.* Vet Immunol Immunopathol, 2014. **157**(1-2): p. 78-86.

54. Asplin, K.E., et al., *Glucose transport in the equine hoof.* Equine Vet J, 2011. **43**(2): p. 196-201.

55. Wagner, I.P., et al., *Evaluation of systemic immunologic hyperreactivity after intradermal testing in horses with chronic laminitis.* Am J Vet Res, 2003. **64**(3): p. 279-83.

56. Steelman, S.M. and B.P. Chowdhary, *Plasma proteomics shows an elevation of the anti-inflammatory protein APOA-IV in chronic equine laminitis.* BMC Vet Res, 2012. **8**(1): p. 179.

57. Vowinkel, T., et al., *Apolipoprotein A-IV inhibits experimental colitis.* J Clin Invest, 2004. **114**(2): p. 260-9.

58. Swaney, J.B., H. Reese, and H.A. Eder, *Polypeptide composition of rat high density lipoprotein: characterization by SDS-gel electrophoresis.* Biochem Biophys Res Commun, 1974. **59**(2): p. 513-9.

59. Weisgraber, K.H., T.P. Bersot, and R.W. Mahley, *Isolation and characterization of an apoprotein from the d less than 1.006 lipoproteins of human and canine lymph homologous with the rat A-IV apoprotein.* Biochem Biophys Res Commun, 1978. **85**(1): p. 287-92.

60. NCBI. *Protein sequence database*. 2013 [cited 2014; Available from: http://www.ncbi.nlm.nih.gov/protein/XP_001502503.1.

61. Wilkins, M.R., et al., *Protein identification and analysis tools in the ExPASy server.* Methods Mol Biol, 1999. **112**: p. 531-52.

62. Green, P.H., et al., *Human apolipoprotein A-IV. Intestinal origin and distribution in plasma.* J Clin Invest, 1980. **65**(4): p. 911-9.

63. Tso, P. and M. Liu, *Apolipoprotein A-IV, food intake, and obesity.* Physiol Behav, 2004. **83**(4): p. 631-43.

64. Wang, F., et al., *Apolipoprotein A-IV improves glucose homeostasis by enhancing insulin secretion.* Proc Natl Acad Sci U S A, 2012. **109**(24): p. 9641-6.

65. Cui, Y., et al., *Genetic ablation of apolipoprotein A-IV accelerates Alzheimer's disease pathogenesis in a mouse model.* Am J Pathol, 2011. **178**(3): p. 1298-308.

66. Qin, X., et al., *Apolipoprotein AIV: a potent endogenous inhibitor of lipid oxidation.* Am J Physiol, 1998. **274**(5 Pt 2): p. H1836-40.

67. Li, T.W., et al., *Screening disease-associated proteins from sera of patients with rheumatoid arthritis: a comparative proteomic study.* Chin Med J (Engl), 2010. **123**(5): p. 537-43.

68. Li, T., et al., *Serum disease-associated proteins of ankylosing spondylitis: results of a preliminary study by comparative proteomics.* Clin Exp Rheumatol, 2010. **28**(2): p. 201-7.

69. Treiber, K.H., D.S. Kronfeld, and R.J. Geor, *Insulin resistance in equids: possible role in laminitis.* J Nutr, 2006. **136**(7 Suppl): p. 2094S-2098S.

70. Rooney, J.R., *Road founder.* Mod Vet Pract, 1978. **59**(5): p. 391-2.

71. Wang, J., *Genome-Wide Transcriptome Analysis of Laminar Tissue During the Early Stages of Experimentally Induced Equine Laminitis.* A Dissertation, 2010.

72. Steelman, S.M., et al., *Cellular and humoral immunity in chronic equine laminitis.* Veterinary Immunology and Immunopathology, 2013. **153**(3-4): p. 217-226.

73. Takahashi, Y., et al., *Enzyme-linked immunosorbent assay for bovine apolipoprotein A-IV.* J Vet Med Sci, 2004. **66**(10): p. 1199-204.

74. Pfaffl, M.W., *A new mathematical model for relative quantification in real-time RT-PCR.* Nucleic Acids Res, 2001. **29**(9): p. e45.

75. Altschul, S.F., et al., *Basic local alignment search tool.* J Mol Biol, 1990. **215**(3): p. 403-10.

76. Bailey, S.R., C.M. Marr, and J. Elliott, *Current research and theories on the pathogenesis of acute laminitis in the horse.* Vet J, 2004. **167**(2): p. 129-42.

77. Rowe, J.B., M.J. Lees, and D.W. Pethick, *Prevention of acidosis and laminitis associated with grain feeding in horses.* J Nutr, 1994. **124**(12 Suppl): p. 2742S-2744S.

78. Malo, C., P. Arsenault, and D. Menard, *Organ culture of the small intestine of the suckling mouse in a serum-free medium.* Cell Tissue Res, 1983. **228**(1): p. 75-84.

79. de Jesus Ribeiro, C., M.T. Ohara, and P. Gama, *Alternative model to human skin organ culture: a preliminary study with Leibovitz L15 medium.* Microsc Res Tech, 2005. **66**(2-3): p. 139-44.

80. Baxter, G.M. and S. Morrison, *Complications of unilateral weight bearing.* Vet Clin North Am Equine Pract, 2008. **24**(3): p. 621-42, ix.

81. Medzhitov, R. and C. Janeway, Jr., *The Toll receptor family and microbial recognition.* Trends Microbiol, 2000. **8**(10): p. 452-6.

82. Chow, J.C., et al., *Toll-like receptor-4 mediates lipopolysaccharide-induced signal transduction.* J Biol Chem, 1999. **274**(16): p. 10689-92.

83. Hoshino, K., et al., *Cutting edge: Toll-like receptor 4 (TLR4)-deficient mice are hyporesponsive to lipopolysaccharide: evidence for TLR4 as the Lps gene product.* J Immunol, 1999. **162**(7): p. 3749-52.

84. Park, B.S. and J.O. Lee, *Recognition of lipopolysaccharide pattern by TLR4 complexes.* Exp Mol Med, 2013. **45**: p. e66.

85. Brodsky, I. and R. Medzhitov, *Two modes of ligand recognition by TLRs.* Cell, 2007. **130**(6): p. 979-81.

86. Akira, S. and K. Takeda, *Toll-like receptor signalling.* Nat Rev Immunol, 2004. **4**(7): p. 499-511.

87. Montes, C.L., et al., *Polyclonal B cell activation in infections: infectious agents' devilry or defense mechanism of the host?* J Leukoc Biol, 2007. **82**(5): p. 1027-32.

88. Noschka, E., et al., *Temporal aspects of laminar gene expression during the developmental stages of equine laminitis.* Vet Immunol Immunopathol, 2009. **129**(3-4): p. 242-53.

89. Moore, J.N., et al., *Intracecal endotoxin and lactate during the onset of equine laminitis: a preliminary report.* Am J Vet Res, 1979. **40**(5): p. 722-3.

90. Arabski, M., et al., *The presence of anti-LPS antibodies and human serum activity against Proteus mirabilis S/R forms in correlation with TLR4 (Thr399Ile) gene polymorphism in rheumatoid arthritis.* Clin Biochem, 2012. **45**(16-17): p. 1374-82.

91. Gripenberg, M., *Common serological features in rheumatoid arthritis and yersinia arthritis. Demonstration of rheumatoid factors and antibodies against ssDNA and Yersinia enterocolitica Lipopolysaccharide by ELISA.* Scand J Rheumatol, 1981. **10**(2): p. 85-91.

92. DiPiro, J.T., et al., *Lipopolysaccharide-reactive immunoglobulin E is associated with lower mortality and organ failure in traumatically injured patients.* Clin Diagn Lab Immunol, 1994. **1**(3): p. 295-8.

93. Wells, M.T., et al., *Anti-LPS antibodies reduce endotoxemia in whole body 60Co irradiated primates: a preliminary report.* Aviat Space Environ Med, 1990. **61**(9): p. 802-6.

94. Kwon, S., et al., *Disparate effects of LPS infusion and carbohydrate overload on inflammatory gene expression in equine laminae.* Vet Immunol Immunopathol, 2013. **155**(1-2): p. 1-8.

95. Hiscott, J., et al., *Characterization of a functional NF-kappa B site in the human interleukin 1 beta promoter: evidence for a positive autoregulatory loop.* Mol Cell Biol, 1993. **13**(10): p. 6231-40.

96. Son, Y.H., et al., *Roles of MAPK and NF-kappaB in interleukin-6 induction by lipopolysaccharide in vascular smooth muscle cells.* J Cardiovasc Pharmacol, 2008. **51**(1): p. 71-7.

97. Libermann, T.A. and D. Baltimore, *Activation of interleukin-6 gene expression through the NF-kappa B transcription factor.* Mol Cell Biol, 1990. **10**(5): p. 2327-34.

98. Kunsch, C. and C.A. Rosen, *NF-kappa B subunit-specific regulation of the interleukin-8 promoter.* Mol Cell Biol, 1993. **13**(10): p. 6137-46.

99. Kang, H.B., et al., *Enhancement of NF-kappaB expression and activity upon differentiation of human embryonic stem cell line SNUhES3.* Stem Cells Dev, 2007. **16**(4): p. 615-23.

100. Shakhov, A.N., et al., *Kappa B-type enhancers are involved in lipopolysaccharide-mediated transcriptional activation of the tumor necrosis factor alpha gene in primary macrophages.* J Exp Med, 1990. **171**(1): p. 35-47.

101. Shakhov, A.N., et al., *Structural analysis of the rabbit TNF locus, containing the genes encoding TNF-beta (lymphotoxin) and TNF-alpha (tumor necrosis factor).* Gene, 1990. **95**(2): p. 215-21.

102. Sica, A., et al., *The c-rel protooncogene product c-Rel but not NF-kappa B binds to the intronic region of the human interferon-gamma gene at a site related to an interferon-stimulable response element.* Proc Natl Acad Sci U S A, 1992. **89**(5): p. 1740-4.

103. Sica, A., et al., *Interaction of NF-kappaB and NFAT with the interferon-gamma promoter.* J Biol Chem, 1997. **272**(48): p. 30412-20.

104. Keyel, P.A., *How is inflammation initiated? Individual influences of IL-1, IL-18 and HMGB1.* Cytokine, 2014.

105. Mills, K.H., et al., *The role of inflammasome-derived IL-1 in driving IL-17 responses.* J Leukoc Biol, 2013. **93**(4): p. 489-97.

106. Black, S.J., et al., *Leukocyte emigration in the early stages of laminitis.* Vet Immunol Immunopathol, 2006. **109**(1-2): p. 161-6.

107. van de Veerdonk, F.L., et al., *Inflammasome activation and IL-1beta and IL-18 processing during infection.* Trends Immunol, 2011. **32**(3): p. 110-6.

108. Romano, M., et al., *Role of IL-6 and its soluble receptor in induction of chemokines and leukocyte recruitment.* Immunity, 1997. **6**(3): p. 315-25.

109. Bickel, M., *The role of interleukin-8 in inflammation and mechanisms of regulation.* J Periodontol, 1993. **64**(5 Suppl): p. 456-60.

110. Zeilhofer, H.U. and W. Schorr, *Role of interleukin-8 in neutrophil signaling.* Curr Opin Hematol, 2000. **7**(3): p. 178-82.

111. Deshmane, S.L., et al., *Monocyte chemoattractant protein-1 (MCP-1): an overview.* J Interferon Cytokine Res, 2009. **29**(6): p. 313-26.

112. Segal, A.W., *How superoxide production by neutrophil leukocytes kills microbes.* Novartis Found Symp, 2006. **279**: p. 92-8; discussion 98-100, 216-9.

113. Brechard, S. and E.J. Tschirhart, *Regulation of superoxide production in neutrophils: role of calcium influx.* J Leukoc Biol, 2008. **84**(5): p. 1223-37.

114. Ueda, A., et al., *NF-kappa B and Sp1 regulate transcription of the human monocyte chemoattractant protein-1 gene.* J Immunol, 1994. **153**(5): p. 2052-63.

115. Ueda, A., et al., *Transcriptional regulation of the human monocyte chemoattractant protein-1 gene. Cooperation of two NF-kappaB sites and NF-kappaB/Rel subunit specificity.* J Biol Chem, 1997. **272**(49): p. 31092-9.

116. Xing, L. and D.G. Remick, *Promoter elements responsible for antioxidant regulation of MCP-1 gene expression.* Antioxid Redox Signal, 2007. **9**(11): p. 1979-89.

117. Xu, Y., et al., *Mutations in the promoter reveal a cause for the reduced expression of the human manganese superoxide dismutase gene in cancer cells.* Oncogene, 1999. **18**(1): p. 93-102.

118. Jones, P.L., D. Ping, and J.M. Boss, *Tumor necrosis factor alpha and interleukin-1beta regulate the murine manganese superoxide dismutase gene through a complex intronic enhancer involving C/EBP-beta and NF-kappaB.* Mol Cell Biol, 1997. **17**(12): p. 6970-81.

119. Kishimoto, T., *IL-6: from its discovery to clinical applications.* Int Immunol, 2010. **22**(5): p. 347-52.

120. Hibi, M., et al., *Molecular cloning and expression of an IL-6 signal transducer, gp130.* Cell, 1990. **63**(6): p. 1149-57.

121. Taga, T., et al., *Interleukin-6 triggers the association of its receptor with a possible signal transducer, gp130.* Cell, 1989. **58**(3): p. 573-81.

122. Rincon, M., *Interleukin-6: from an inflammatory marker to a target for inflammatory diseases.* Trends Immunol, 2012. **33**(11): p. 571-7.

123. Silvennoinen, O., et al., *Interferon-induced nuclear signalling by Jak protein tyrosine kinases.* Nature, 1993. **366**(6455): p. 583-5.

124. Wegenka, U.M., et al., *Acute-phase response factor, a nuclear factor binding to acute-phase response elements, is rapidly activated by interleukin-6 at the posttranslational level.* Mol Cell Biol, 1993. **13**(1): p. 276-88.

125. Zhong, Z., Z. Wen, and J.E. Darnell, Jr., *Stat3: a STAT family member activated by tyrosine phosphorylation in response to epidermal growth factor and interleukin-6.* Science, 1994. **264**(5155): p. 95-8.

126. Tadros, E.M., et al., *Effects of a "two-hit" model of organ damage on the systemic inflammatory response and development of laminitis in horses.* Vet Immunol Immunopathol, 2012. **150**(1-2): p. 90-100.

127. Ostos, M.A., et al., *Antioxidative and antiatherosclerotic effects of human apolipoprotein A-IV in apolipoprotein E-deficient mice.* Arterioscler Thromb Vasc Biol, 2001. **21**(6): p. 1023-8.

128. Hansel, B., et al., *Alterations in lipoprotein defense against oxidative stress in metabolic syndrome.* Curr Atheroscler Rep, 2006. **8**(6): p. 501-9.

129. VerHague, M.A., et al., *Apolipoprotein A-IV expression in mouse liver enhances triglyceride secretion and reduces hepatic lipid content by promoting very low density lipoprotein particle expansion.* Arterioscler Thromb Vasc Biol, 2013. **33**(11): p. 2501-8.

130. Hanniman, E.A., et al., *Apolipoprotein A-IV is regulated by nutritional and metabolic stress: involvement of glucocorticoids, HNF-4 alpha, and PGC-1 alpha.* J Lipid Res, 2006. **47**(11): p. 2503-14.

131. Wu, A.L. and H.G. Windmueller, *Relative contributions by liver and intestine to individual plasma apolipoproteins in the rat.* J Biol Chem, 1979. **254**(15): p. 7316-22.

132. Li, X., et al., *Apolipoprotein A-IV reduces hepatic gluconeogenesis through nuclear receptor NR1D1.* J Biol Chem, 2014. **289**(4): p. 2396-404.

133. Kalogeris, T.J., K. Fukagawa, and P. Tso, *Synthesis and lymphatic transport of intestinal apolipoprotein A-IV in response to graded doses of triglyceride.* J Lipid Res, 1994. **35**(7): p. 1141-51.

134. Hayashi, H., et al., *Transport of lipid and apolipoproteins A-I and A-IV in intestinal lymph of the rat.* J Lipid Res, 1990. **31**(9): p. 1613-25.

135. Fukagawa, K., et al., *Circadian rhythm of serum and lymph apolipoprotein AIV in ad libitum-fed and fasted rats.* Am J Physiol, 1994. **267**(5 Pt 2): p. R1385-90.

136. Apfelbaum, T.F., N.O. Davidson, and R.M. Glickman, *Apolipoprotein A-IV synthesis in rat intestine: regulation by dietary triglyceride.* Am J Physiol, 1987. **252**(5 Pt 1): p. G662-6.